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PRINCIPAL INVESTIGATOR: Donna H. Ryan, M.D.

CONTRACTING ORGANIZATION: Louisiana State University
Baton Rouge, Louisiana 70803-1800

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To assess, maintain, or improve a soldier's physical/physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health safety and performance, the Pennington Biomedical Research Center (PBRC) performs the following six research tasks: 1) The Clinical Laboratory for Human and Food Samples performs laboratory analysis of samples from studies conducted by the U.S. Army Research Institute of Environmental Medicine (USARIEM). 2) The Stable Isotope Laboratory performs analyses to measure the energy expenditure and body composition of soldiers during prolonged field exercise. 3) The Nutritional Neuroscience Laboratory conducts multi-disciplinary basic research studies on the effects of diet on biochemical, morphologic and behavioral variables. The lab evaluates the role of diet in sustaining performance under conditions of stress and sleep deprivation. 4) The Nutritional Neuroscience Clinical Studies evaluate nutritional and other strategies to optimize cognitive performance in conditions of operational stress and sleep deprivation. 5) The Menu Modification project evaluates and improves garrison meals at an actual garrison in Fort Polk, Louisiana. 6) The Metabolic Unit Project allows for the PBRC's inpatient unit to conduct metabolic studies of military relevance in an inpatient setting.

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FOREWORD

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Donna H. Ryan, M.D.
Principal Investigator

May 1, 1997
Date

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Introduction

On April 1, 1992, Grant #DAMD 17-92-V2009 was awarded to Pennington Biomedical Research Center (PBRC) to address the following **Hypothesis: Medical factors limiting soldier effectiveness can be addressed through nutritional strategies**.

The goal of this research is to assess, maintain, or improve a soldier's physical/physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health, safety and performance.

Technical Objective

This research continues the research relationship between the PBRC and USARIEM over a five year period. Those research relationships were established under a prior cooperative agreement, #DAMD 17-88-Z-8023, "The effect of food, diet and nutrition on military readiness and preparedness of military personnel and dependents in a peace time environment."

The project allows for the continuation of the Clinical Laboratory for Human and Food Samples, Stable Isotope Laboratory, Menu Modification Project, and Nutritional Neuroscience Laboratory, all of which were initiated under Grant #DAMD 17-88-Z-8023. The project also expands the scope of research to involve Nutritional Neuroscience Clinical Studies. The project also allows for the utilization of the PBRC's inpatient metabolic unit for a study designed by USARIEM investigators as detailed in the Metabolic Unit Project section.

The six tasks performed under this project are listed and described below.

Task 1: Clinical Laboratory for Human and Food Samples

The Clinical Laboratory performs procedures (assessment of protein, mineral, vitamin and immunologic status) to assess the nutritional status of soldiers participating in military nutrition research studies conducted by USARIEM. In the year covered in this annual report, the Clinical Laboratory took on three research projects sponsored by the Army Defense Women's Health Initiative.

Task 2: Stable Isotope Laboratory

The Stable Isotope Laboratory continues the development and field validation of stable isotope technologies to unobtrusively measure the energy expenditure of soldiers during prolonged (1-4 weeks) field exercise in extreme climates. The technology also measures changes in body

composition and body fluid status.

Task 3: Nutritional Neurosciences Laboratory

Continuation of the research in the Nutritional Neuroscience Laboratory includes multi-disciplinary studies in rats on the effect of diet on brain function and structure. The biochemical and morphologic variables are related to changes in behavior measured by arousal, shuttle box performance, operant chamber performance, food selection and swimming performance. The effect of stress and in particular the stress of rapid eye-movement sleep deprivation are studied using the above noted morphologic and behavioral parameters. Mechanistic hypotheses are explored and ameliorative dietary strategies are tested.

Task 4: Nutritional Neurosciences Clinical Studies

The Nutritional Neuroscience Clinical Studies are designed to evaluate cognitive performance and man-machine interface under conditions of sleep deprivation. Different nutritional intervention strategies to favorably influence mental performance in conditions of rapid eye-movement sleep deprivation are tested.

Task 5: Menu Modification Project

PBRC nutritionists evaluate, through a computer data base, the Moore's Extended Nutrient Database (MENU), and laboratory analyses, the nutritional content of garrison meals. In a project conducted at Fort Polk, Louisiana, menus are modified and tested in an actual garrison setting to meet the improved Army guidelines for nutrition. Follow up of the testing includes further modifications and testing, using the quality improvement approach. PBRC personnel travel to field sites for assistance in ARIEM-directed studies of food intake in the field.

Task 6: Metabolic Unit Project

The PBRC inpatient 14 bed unit is made available for research studies and has accommodated Special Operation Forces volunteers in a research study designed by a collaboration of USARIEM scientists and participation by PBRC personnel. No activities in 96-97 occupied this facility.

Military Significance and Relevance to USARIEM Needs

The Stable Isotope and Clinical Laboratory methodologies are critical components of in-house military nutrition research of the U.S. Army Research Institute of Environmental Medicine. These extramural projects provide critical capabilities that do not exist in house, but are needed to fulfill the Army Surgeon General's responsibility to provide nutritional research support to the DOD and Nutrition RDT&E Program.

The Clinical Laboratory also provides support for three projects of Defense Women's Nutritional Health Research.

The Nutritional Neuroscience Laboratory and Clinical Studies Programs expand our knowledge of the effects of stress and sleep deprivation and explores the ameliorative effects and mechanisms of action of dietary-induced alterations in behavior and cognitive function. Advances in this knowledge are the basis for developing safe and effective nutritional strategies to sustain and enhance soldier performance under conditions of environmental or operational stress. The project also provides insight into the roles of corticotrophin releasing factor (CRF) and locus coeruleus (LC) noradrenergic mechanisms in mediating anxiety in rats exposed to restraint stress.

The Menu Modification Project fulfills military needs to promote health, maintain readiness and sustain soldier performance.

The Metabolic Unit Project fulfills military need for an inpatient site for performance of specialized research utilizing the body composition assessment, energy expenditure assessment, metabolic kitchen services, and clinical laboratory expertise of the PBRC.

This annual report describes progress during the fifth year of the grant.

The major administrative change in this year was the planned extension of grant activities. At a site visit of the Committee for Military Nutrition Research (CMNR), which is described in detail later, plans were presented to extend the work into 1997-1998. These plans were approved as were plans for a renewed effort whereby some tasks will be continued in a new grant activity.

The major administrative and scientific high points of the fifth year are outlined below:

- On Monday, May 20 and Tuesday, May 21, 1996, Donna Ryan, Jeff Zachwieja and Jennifer Rood attended the CMNR Workshop, "Nutrition and Immune Function: Strategies for Sustainment in the Field."

- We received notification that our annual report submitted in May, 1996 for the fourth year of the project was reviewed and was acceptable as written. A letter documenting this was dated July 10, 1996 and is found in the 18th Quarterly Report.

- On July 19, 1996 we received a modification of our cooperative agreement to incrementally fund the remaining amount of Option Year 4 and to provide complete funding for work on projects that were limited over the past two years due to shortfall funding. That funding amounted to $1.683 million. A copy of that modification can be found in the appendix of the 18th Quarterly Report.

- On July 8, 1996 we were visited by Project Officer, Dr. Harris Lieberman. The purpose of that visit was to refine the nutritional neuroscience projects. At that time we began

planning a project to look at stress and immune function that would interdigitate with the nutritional neuroscience projects. Details of that planning are found in the 18th Quarterly Report.

- On August 9, 1996 we were visited by Dr. Harris Lieberman and Colonel Karl Friedl. An agenda for that visit is found in the 18th Quarterly Report. The purpose of that visit was to review the preproposal and the plans for the visit of the CMNR.

- On September 18-20, 1996 the PBRC hosted a site visit for the CMNR. On Wednesday, September 18 the PBRC faculty presented proposed research activities for the site visitors. On September 19-20 the CMNR met at the PBRC Conference Center and pursued their own agenda. Details of the site visit agenda are found in the 18th Quarterly Report. The members who attended the meeting included the following:

  Robert O. Nesheim, Ph.D., Chair
  William R. Beisel, M.D.
  Gail K. Butterfield, Ph.D.
  John D. Fernstrom, Ph.D.
  G. Richard Jansen, Ph.D.
  Robin B. Kanarek, Ph.D.
  Orville Levander, Ph.D.
  John Vanderveen, Ph.D.

  The CMNR staff who attended included:

  Rebecca Costello, Ph.D.
  Sydne Carlson-Newberry
  Susan Knasiak
  Donna F. Allen

- The report of the CMNR site visit was published on November 21, 1996 and submitted to Brigadier General Russ Zajtchuk, Commander, U.S. Army Medical Research and Materiel Command. The letter report of the CMNR site visit can be found in the appendix to the 19th Quarterly Report.

- As a result of the CMNR review and as a result of the continual planning efforts for this fiscal year, we made two major modifications to our research plan. First, a proposed protocol, "Effects of Prolonged Inactivity of Musculoskeletal and Cardiovascular Systems with Evaluation of a Potential Countermeasure," was deferred. This activity was not funded by Army grant monies because of the CMNR report stating, "This area of research appears to be of greater importance for NASA than the Army and further development and support should be provided by NASA."

- The second major alteration in the overall research plan was to discontinue further sleep studies following their completion in this fiscal year. The major activities in fiscal year

1996-1997 with regards to the clinical neuroscience projects have been in completing the protocol and analyzing the data for publication.

- An additional issue that arose from the CMNR report was the development of projects would address the issue of stress, physical performance, mental performance, immune function and their interrelationships with nutritional factors. Initial planning began to integrate these topics into future research to be conducted for the Army. Details of this are found in the presentation to the CMNR site visit and in the CMNR report found in the 18th and 19th Quarterly Reports respectively.

- On February 5, 1997 we received correspondence from Judy Pawlus, Chief, Research Data Management, informing us of the Army's intent to downgrade our reports from limited to unlimited, approved for public release, status. This correspondence can be found in the 20th Quarterly Report.

- On March 18, 1997 we received notification from Wendy A. Cockerham, Procurement Technician, of a modification to our cooperative agreement that allowed us a no cost extension of one year to enable us to complete approved projects. This document is found in the appendix of the 20th Quarterly Report.

- We received confirmation that the final report for this project is to be submitted in 1998. Correspondence documenting this is found in the appendix of the 20th Quarterly Report.

- On February 7, 1997 we received confirmation from Lt. Col. Alana Cline that she will be joining the PBRC in May, 1997 to continue the Menu Modification Project. A copy of this correspondence is found in the 20th Quarterly Report.

- On Thursday, March 19, 1997, a workshop was held at the Foundry Building in Washington, D.C. entitled, "The Role of Protein and Amino Acids in Sustaining and Enhancing Military Performance." Drs. Jim DeLany, Jeff Zachwieja and Cathy Champagne represented the PBRC at this meeting. Dr. Alana Cline was one of the speakers.

- Throughout 1996-1997 PBRC personnel served as research support people to military nutrition studies in the field. These activities are documented in the quarterly reports and represent a change in status for the conduct of these research studies.

- In order to support the planned expansion of services to be provided under our next Army grant, we arranged for additional equipment to be purchased to support the laboratories of Dr. Jeff Zachwieja and Dr. David Horohov. These laboratories will evaluate immunologic assessment of humans and animals (Horohov) and the role of stress, nutrition and physical performance (Zachwieja). Documentation of this activity can be found in the 20th Quarterly Report.

- The response to the CMNR report was finalized in the last quarter of this year and that response can be found in the appendix of the 20th Quarterly Report.

Discussions of individual projects funded under this grant follow.

I. Clinical Laboratory for Human and Food Samples

A. Overview

The Clinical Research Laboratory, which functions as a support laboratory for the U.S. Army's nutritional research program, continued to receive and analyze samples for the Army in 1996-1997. During this past year, a total of eleven projects were worked on and a total of 24 people were sent to assist in sample processing on field projects for three studies, each of which had multiple draws. The laboratory now employs two clinical chemist, three accessioners, one phlebotomist, four students, eight full time and three part-time ASCP registered medical technologists. The Food Analysis Laboratory employs one Masters' level food chemist, one B.S. level Research Associate, two undergraduate students, and one graduate student.

Ken Smith took over the duty of Business Manager in 1997 and Deonne Bodin became Research Facilitator, replacing Joanie Wilson and Mendy Richard, respectively.

B. Progress on Completed Projects

The Clinical Research Laboratory continued to support research conducted by USARIEM. Studies from which samples were analyzed included the SOF study, the Sleep Deprivation Study, the Ranger 3 study (four blood draws), the Sergeant Major's Academy Nutritional Survey (three blood draws), the Menu Modification Study, the Ranger Regiment Nutritional Survey, the SAFS-5 study, and the SAFS-6 study (two blood draws). Also, Women's studies from which samples were analyzed included the Return to Fitness Study, the IDNS study, and the WISP study. The majority of analyses for these studies have been completed. Only a few tests for a few studies are pending (see section 3 below).

Representatives from the Clinical Laboratory attended blood draws for the Ranger 3 (four draws), Sergeant Major Academy Nutritional Survey (three draws), SAFS-6 (two draws), the Ranger Regiment Nutritional Survey (one draw), and IDNS study (one draw).

The Food Analysis Laboratory analyzed total and soluble dietary fiber for thirteen foods for the SAFS-5 study.

Research and development saw the implementation of a method for the analysis of amino acids by HPLC (see appendix). In addition, a method for pyruvate on microtiter plates was developed and a year long study on sample stability was completed. Results of this study will be

forthcoming pending data analysis.

C. Progress on Ongoing Projects

Studies which were ongoing include the SAFS-6 study in which samples have yet to be received from Natick. Analyses will commence once those samples have been received. In addition, some tests have not been completed for a few studies. These include the SAFS-5 (vitamins A, E), Sergeant Major's Academy (homocysteine, vitamins A&E), Ranger Regiment Nutritional Survey (SAFS-5) (Vitamin A, E, Homocysteine), Banderet Tyrosine study (tyrosine, cortisol, catecholamines), SOF2 study (RBC Enzymes), Ranger 3 study (amino acids), and Hot Weather Feeding Study (RBC Enzymes). Technical difficulties have encumbered these studies. Vitamins A&E are now being analyzed but were backlogged because of instrumental difficulties and personnel shortages. Some samples from the Sergeant Major's Academy study draw one, earmarked for the analysis of vitamins A&E, had to be returned to Natick because of shortages of samples for cytokine analysis. Homocysteine method development was suspended due to personnel and instrumental priorities but will soon be on track again. Supplies for the development of a method for homocysteine on the capillary electrophoresis system have been ordered and received. Research has begun on the correlation of plasma catecholamines by radioimmunoassay versus HPLC. Provided adequate results are obtained, the implementation of a method by radioimmunoassay should relieve the burden imposed on the HPLC analysis, which is slow and cumbersome. Cortisol analysis is continuing and will soon be completed. Tyrosines will begin as soon as the Ranger 3 amino acids are completed.

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II. Stable Isotope Laboratory

A. Overview

The research conducted by the Stable Isotope Laboratory is in the area of energy and water requirements, and changes in body water, of soldiers under harsh environmental conditions. The method used to determine energy requirements is the doubly labeled water technique, which involves oral administration of water labeled with the stable isotopes, ^{2}H and ^{18}O. Saliva and urine samples are then obtained for periods of 4-14 days, longer with redosing. Water intake can be determined using only the ^{2}H labeled water.

The use of doubly labeled water for measurement of energy expenditure was developed as a field technique for use in small animals (1). The method is based on the premise that after a loading dose of $^{2}H_{2}{}^{18}O$, ^{18}O is eliminated as CO_2 and water, while deuterium is eliminated from the body as water. The rate of CO_2 production, and, hence, energy expenditure, is calculated from the difference of the two elimination rates. Doubly labeled water, using the two-point method, is an ideal method for use in free-living subjects because it is noninvasive and nonrestrictive. The only requirement of subjects is to give urine and saliva specimens before and after drinking an initial dose of $^{2}H_{2}{}^{18}O$, and then return in one to two weeks to give a final urine specimen. During the period between the two urine and saliva samplings, subjects are free to carry out their normal activities and

are not required to maintain extensive diaries.

The doubly labeled water method has been extensively validated in humans under controlled settings (2), but there are confounding factors that need to be considered in field studies, particularly in Army Field Studies. Among these are change in location or food and water supply immediately preceding, or during an energy expenditure study. These changes may cause a change in baseline isotope abundance and, therefore, interfere with the accuracy of the energy expenditure measurement. This has occurred in a previous field training exercise involving the study of the ME. and R.W. rations (3). This is a particular problem with studies such as the Ranger Training Studies (4), in which soldiers are moved to different parts of the country during the study. Therefore, a group not receiving labeled water must be followed to make any corrections in baseline isotope shifts.

Hydration status is another main focus for some Army studies. Using the cheaper and more readily available deuterium tracer, either changes in total body water (5,6) can be followed during a study, or water turnover (intake) (7,8) can be measured during a study.

The Stable Isotope Lab was involved in several Army research projects during the current year. These are described below.

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B. Progress on Ongoing Projects

Two recent projects were conducted in the USARIEM altitude chambers. These accumulated samples were sent to the Stable Isotope Lab for analyses. These two studies were the Acute Mountain Sickness Glycerol (ACM) 95 study on men and the Women at Altitude 95 study on AMS and the menstrual cycle. These two studies are outlined below:

1. Women at Altitude 95: PI, LTC Rock
   160 saliva samples for deuterium analyses

   This study included samples from three different phases of the menstrual cycle; early follicular, late follicular and mid-leuteal. Four samples were taken for each period, a 6 am and 10 am sample at sea level and a 6 am and 10 am sample after 24 hours at altitude. Therefore there were 12 samples for each subject. Isotope analyses for calculation of total body water were completed. A summary Table was included in the 17th Quarterly Report, with raw data presented in the Appendix of that Report.

2. Acute Mountain Sickness 95: PI, CPT Lyons
   94 saliva samples for deuterium and bromide analyses

   This study includes six samples per subject per trial, with placebo or with experimental glycerol solution, for total body water by deuterium dilution and extracellular water by bromide dilution. Samples were taken at 6 am and 10 am after an overnight fast at sea level, high altitude day 2 and high altitude day 4. Thus there are 12 samples for each subject. Isotope analyses for this study have been completed and given in the following Table.

	Trial 1			Trial 2		
Subj.	**Sea Level**	**day 2 H. Altitude**	**day 4 H. Altitude**	**Sea Level**	**day 2 H. Altitude**	**day 4 H. Altitude**
1	1931.5					
2	1696.8	1769.6	2264.7	886.1	2257.2	1584.4
3	1846.8	1807.7	1838.2	1678.7	1862.9	1861.2
4	2417.9	1664.7	2296.1	2340.8	1949.9	2374.4
5	1636.7	1785.7	1697.4			

6	1623.3	960.6	589.4	1663.6	1074.7	
7	1806.9	1731.9	1752.0	1909.9	1744.2	1097.1
8	1821.6	2073.1	1832.2	1775.5	452.2	3110.8
9						
10	1707.9	1751.9		1672.0	1694.5	
11	1922.8					
12	2189.6	1730.2	2022.2			

The isotope analyses for the Greenland Expedition are complete. The energy expenditure values for the two subjects by period are given in the table below. There was a problem with the later samples (13 and 15 day) for the third period for both subjects. The raw data are presented in the Appendix.

Energy Expenditure, kcal/d

		Subject	
Period	day	R	T
1	7	3250	3380
	13	4700	5350
	15	5250	6170
2	7	3970	3900
	11	3790	3600
	13	3750	3790
3	7	4713	4940

The isotope analyses for the Norwegian Ranger Study are complete. The deuterium and Oxygen-18 baseline isotope shifts for the two placebo groups (See Table below) were calculated to correct the isotope data for the labeled subjects.

Placebo Summary

	Deuterium			O-18		
	#6	#12	avg.	#6	#12	avg.
6/18	-1.6		-1.6	-0.12		-0.12
6/24	-8.5	-12.5	-10.5	-1.39	-2.43	-1.91
6/25	-13.6	-15.1	-14.4	-1.57	-2.64	-2.11

The energy expenditure values for the two subjects by period are given in the table below. The raw data for TBW determinations and deuterium and O-18 elimination rates are presented in the Appendix of the 20th Quarterly Report.

	Energy Expenditure	
Subject	06/24/96	06/24/96
1	5324	6373
2	6423	6329
3	6440	6211
4	4908	5114
5	6060	5926
7	5710	5905
8	5042	5441
9	6394	7229
10	6486	6940
11	6625	6850
Mean	5941	6232
SD	648	665

Isotope analyses are complete for the Ranger 96 Study and final calculations are nearly complete.

C. Progress on Ongoing Projects

Isotope analyses are complete for the Ranger 96 Study and final calculations are nearly complete.

Dr. DeLany is making progress on his DWHRP Grant, "Determination of Total Daily Energy Requirements and Activity Patterns of Servicewomen," in collaboration with Harris Lieberman, Karl Friedl and Reed Hoyt.

D. Manuscripts Published/In Press

Dr. DeLany submitted a chapter, "Doubly-Labeled Water for Energy Expenditure," for the book "Emerging Technologies For Nutrition Research: Potential For Assessing Military Performance Capability." This is based on a workshop sponsored by the Committee on Military Nutrition Research.

Lyons TP, SR Muza, A Cymerman, JP DeLany. Total body water and plasma volume responses during high altitude acclimatization and upon return to sea level. Submitted, Int J Sports Medicine, 1996.

E. Manuscripts in Preparation

DeLany, James P, Robert J Moore, Andrew Young, Nancy King, John Edwards, Reed W Hoyt and E Wayne Askew. Energy expenditure during field training in an Arctic environment. Rejected, Revision and resubmission underway with addition of activity data with Reed Hoyt.

DeLany JP, RW Hoyt, EW Askew. Energy expenditure of unacclimatized soldiers working at altitude. Manuscript being revised to add activity data with Reed Hoyt.

Hoyt RW, RJ Moore, JP DeLany, KE Friedl and EW Askew. Energy balance during 62 days of rigorous physical activity and caloric restriction. In Preparation, 1997.

III. & IV. Nutritional Neuroscience Laboratory: Basic and Clinical Studies

Task 3 - Basic

A. Overview

There have been several changes in personnel in the Basic Neuroscience group this year. In June, You (Joe) Zhou joined the group as an Instructor. His molecular biology expertise has enabled us to initiate studies to identify potential genetic markers for stress responsiveness. Igor Rybkin has been accepted to the graduate program at the Medical School in San Antonio and will be leaving to start his studies at the end of May, 1997. Jun Zhou, who has worked as a Research Associate on the project for 3 years, is now a graduate student and her Ph.D. thesis will focus on the mechanisms responsible for stress-induced anorexia. In January, Leigh Anne Howell joined the laboratory as a Research Associate. Leigh Anne is experienced in animal behavior techniques and supports many of the studies in progress.

We have made significant progress on a number of projects this year. Joe has been focusing on Apolipoprotein E (Apo E) as a potential marker for stress responsiveness. He has established several behavioral methodologies for mice, has demonstrated that Apo E expression is increased in response to chronic stress and that transgenic ApoE knock-out mice have a substantially impaired spatial memory, similar to that observed in normal mice exposed to repeated stress. Gennady Smagin has developed antisense techniques that will enable us to identify proteins and receptors essential for mediation of stress-induced behavioral responses. He has been able to prevent CRF and urocortin induced hypophagia by selective suppression of CRF1 and CRF2 receptors. Igor Rybkin and Joe Zhou developed a probe for urocortin and are in the process of determining the effects of stress on expression of this protein, which may mediate some of the behaviors observed in stressed rats.

We conducted two studies intended to identify the importance of serotonin metabolism in mechanisms that impair spatial memory in sleep deprived rats. In the first experiment the diet was supplemented with tryptophan, the precursor for serotonin, and in the second study the diet

was supplemented with leucine, an amino acid that competes with tryptophan for transport across the blood brain barrier. Although we found that leucine had significant effects on memory of non-stressed control rats, we were unable to modify the stress-induced changes in spatial memory or central concentrations of tryptophan. It was concluded that the control diet contained so much high quality protein that it was preventing meaningful supplementation of dietary amino acids. We have now identified the minimal amount of amino acids that support normal growth in adult male rats and are currently testing a newly formulated liquid diet for efficacy. If the rats find this diet acceptable it will be used in future studies and should optimize our ability to identify beneficial effects of supplementing the diet with specific amino acids that are precursors for neurotransmitters.

We have focused a significant amount of attention on the effects of restraint stress on food intake and body weight. Igor demonstrated that a single 3 hour exposure to restraint caused a prolonged suppression of body weight which was more severe if stress was applied in the morning than in the evening. Examination of neurotransmitters known to medicate the acute effects of stress on feeding were examined but we were unable to identify the mechanisms responsible for the sustained suppression of food intake, which was apparent 9 hours after the end of stress. In other studies we have found that food intakes of rats exposed to repeated restraint is inhibited for 5 days after the end of stress and that the rats do not return to the weight of non-stressed controls even after 40 days. In collaboration with Dr. David Horohov we have found that immune function of the restrained rats is significantly impaired if the restraint is applied in conjunction with feeding a high-fat diet. In addition, rats fed the high fat diet selectively lose lean tissue. We intend to follow up on these observations to determine whether the fatty acid composition of the diet influences the stress-induced effects on energy balance and immune function.

Finally Bradley Youngblood has developed a model in which restraint stress is combined with partial water immersion to induce an impairment in operant function of rats that have been trained to level press for food in an FR5 schedule. As the stress does not disrupt free feeding behavior of the rats he has concluded that the impairment of operant behavior is secondary to loss of motor control which may be caused by central catecholamine depletion. Studies are in progress to determine whether pre-treatment of the rats with a catecholamine precursor, tyrosine, prevents the stress-induced impairment of motor function.

Taken together these studies demonstrate a very successful and productive year on this project. This is also reflected by the number of manuscripts and abstracts that have been presented by this group this year.

B. Progress on Completed Projects

The Effect of Dietary Vitamin E on Stress-Induced Behaviors

Jun Zhou

It is known that stress can impair immune function (Keller et al., 1981) and that antioxidants can improve immune function (Bendich et al., 1986; Meydani, 1995) and may also protect against exercise induced oxidative stress (Goldfarb, 1992). In stressed animals corticosterone increases catecholamine, serotonin (5-HT) and 5-hydroxyindolacetic acid (5-HIAA) concentration in many areas of the brain including the hypothalamus, hippocampus and cortex (Jhanwar-Uniyal et al., 1987; Hastings et al., 1996). Increased metabolism of these neurotransmitters has been associated with oxidative damage and neuronal degeneration (Hasting et al., 1996) and has been implicated in the development of several neuro-degenerative diseases that occur with increased frequency during aging (Coyle and Puttfarcken, 1993). Based on these observations the following study examined the effect of dietary antioxidant on behavioral responses in stressed rats. In order to determine the effect of dietary supplementation on responses to a variety of situations the animals were exposed to a series of stressors which influenced different types of behavior.

Methods and Results

Male Wistar rats (300g) were fed ad libitum one of three diets containing different concentrations of the antioxidant, vitamin E (30 iu, 75 iu or 150 iu/Kg). The diets were a tocopherol-free diet (ICN) supplemented with (+)-α-tocopherol acid succinate. Following 5 weeks adaptation to diets daily measurements of food intake and body weight were initiated. Three different stress models were used in the following sequence: (1) The effect of acute 3 hr. restraint stress on open field activity, food intake and body weight. (2) The effect of 48 hours of REMd on spatial memory and body weight. (3) The effect of acute one hour restraint-water immersion stress on neurotransmitter concentration in the hypothalamus and serum corticosterone. Thymus and spleen weights were recorded at the end of last experiment.

Experiment I: The effect of acute restraint stress on open field activity, food intake and body weight. The three groups of rats were restrained in plastic restrainers for three hours in a counter-balanced sequence. Immediately following restraint, they were tested for open field activity for 5 minutes. There was no significant effect of dietary Vitamin E concentration on any parameter measured.

Table 1. Open field activity of rats fed diets containing different concentrations of Vit E

		1 st Minute				5 Minutes		
	Latency	Outside Sq	Inside Sq	Total Sq	Rearing	Total Sq	Outside Sq	Inside Sq
30 iu/Kg	22 ± 22	9 ± 7	0.4 ± 0.8	9 ± 7	4 ± 3	53 ± 25	49 ± 25	4 ± 4
75 iu/Kg	20 ± 34	12 ± 6	0.1 ± 0.4	12 ± 6	4 ± 2	60 ± 9	57 ± 6	4 ± 4
150 iu/Kg	20 ± 13	9 ± 5	0.3 ± 0.8	9 ± 6	4 ± 2	62 ± 22	61 ± 21	2 ± 2

Data are means ± sd for groups of 7 rats. There was no effect of diet on the open field activities of the rats following 3 hours of restraint stress.

Restraint caused a significant reduction in food intake, compared with baseline, but there was no significant effect of dietary Vit E content. There was no relationship between the reduction in food intake and Vit E content of the diet. Restraint caused significant loss of the body weight, compared with baseline, but there was no significant effect of dietary Vit E.

Experiment II: The Effect of 48 Hours of Rapid Eye Movement sleep deprivation (REMd) on Spatial Memory, Food Intake and Body Weight. The rats were divided into two groups: 48 hours of REMd induced by the "flower-pot" technique (Mendelson et al., 1974) or control. The REMd animals were housed on small platforms in a water tank and the control animals were housed individually in shoe-box cages. Rats in both groups had free access to chow and water.

On day 1 and day 2 of REMd, working and reference spatial memory were measured using a Place-learning Set task (Auer et al., 1989) in a Morris Water Maze. After the second day of testing, the rats were returned to hanging wire mesh cages and the Vit E diets. Two weeks later, the experiment was repeated, but the animals that had been exposed to REMd were switched to the control group and the rats had been controls were switched to the REMd group. In this way, each animal was tested twice: once in the REMd condition and once as a control. Statistically significant difference between groups were determined by two way ANOVA, for spatial memory test and by repeated measures for body weight.

The results from spatial memory test are show in Figure 1. After 24 hours REMd, sleep deprived rats swam further to locate the platform than control rats in the first trial. Therefore, REMd caused a significant impairment of reference spatial memory ($P<0.01$), however, there was no significant effect of dietary Vit E concentration on reference memory. No significant difference was found on rats working memory, comparing REMd with control rats or comparing the three Vit E levels in the diet. After 48 hours of REMd, there was no significant effect of sleep deprivation on reference memory while the rats fed the 150 iu/kg Vit E diet group had an impaired reference memory compared with the other groups ($P<0.05$). As for working memory,

there were significance effects of sample (different levers of Vit E), treatment (REMd and control) and a significant interaction (see Figure 1). Significance was found between the REMd and control groups ($p<0.01$) and within the REMd group between rats fed 30 iu/kg Vit E and 75 iu/kg Vit E diets ($p<0.01$). This result indicates that the impairment of working memory caused by REMd can be minimized by a diet containing the recommended dose of Vit E.

Experiment III: The effect of acute one hour restraint-water immersion stress on neurotransmitter contents, weight of thymus and weight of spleen. After 12 weeks of feeding diets containing three different levels of Vit E, the rats in 30 iu/kg and 150 iu/kg groups were put into restrainers and partially immersed in water for 1 hour. The 75 iu/kg group was used as the control and was not subjected to stress. At the end of restraint-water immersion, the rats were decapitated. Blood was collected for measurement of serum corticosterone. Hypothalamus, thymus and spleen were dissected, weighed and frozen. The hypothalamus was homogenized in 0.1M perchloric acid and neurotransmitter content was measured by HPLC with electrochemical detection.

The results for final body weight, weights of thymus and spleen are summarized in Table 2. There was no significant difference for any measurement except serum corticosterone, ($P<0.01$ stressed rats compared with control).

Table 2: Measurements in Vit E diet rats

	30 iu/kg Vit	75 iu/kg Vit	150 iu/kg Vit
Beginning Body	401.3±10.4	413.0±10.8	410.7±18.5
Ending Body Weight(g)	500.3±19.5	524.7±15.0	538.4±22.8
Changed Body	99.0±10.0	111.7±7.1	127.7±5.9
Thymus (mg)	490.8±19.7	538.5±69.5	489.3±43.0
Thymus/body weight	0.99±0.06	1.01±0.1	0.91±0.07
Spleen (mg)	838.8±50.4	964.4±63.1	807.1±39.9
Spleen/body weight	1.96±0.11	1.83±0.1	1.51±0.08
Corticosterone (ng/ml)	1155.7±198*	214.3±45.7	916.0±96**

Data are means ± sem for groups of 7 rats . **: $P<0.01$ compared with the 75 iu/kg group

Concentrations of neurotransmitters in hypothalamus are shown in Table 3. There were significant differences in the contents of DOPAC, HIAA and HVA in the 30 iu/kg Vit E group compared with the control 75 iu/kg Vit E group ($P<0.01$ or $P<0.05$). These differences were not present in the 150 iu/kg group. The only difference in the 150 iu/kg group was in HVA content, compared with the 30 iu/kg group. Although 5-HIAA, a metabolite of 5-HT, DOPAC and HVA, metabolites of dopamine, were significantly increased in rats fed the lower dose of Vit E, there was no significant change in metabolism of either dopamine or 5-HT.

Table 3. Hypothalamic neurotransmitter concentration and metabolism in rats fed diets of different Vit E content

	30	75	150
	0.92 +	1.06 +	1.03 +
DOPAC	0.17 +	0.10 +	0.15 +
	0.56 +	0.39 +	0.42 +
	0.34 +	0.29 +	0.37 +
HIAA	0.32 +	0.24 +	0.27 +
HVA	0.09 +	0.04 +	0.06 +
5-HT	0.29 +	0.23 +	0.21 +
HIAA/5-	1.22 +	1.12 +	1.40 +
	0.17 +	0.12 +	0.16 +

Data re means $\pm$ sem for groups of 7 rats. Statistical significance : ** $P<0.01$ compared with 75 iu/kg group, * $P<0.05$ compared with 75 iu/kg group, # $P<0.05$ compared with 30 iu/kg group.

Conclusions

The results from this experiment suggest that varying dietary Vit E concentration above or below NRC recommended levels (75 iu/Kg) influences the behavioral response to chronic stress. There was no effect of diet composition on measures of anxiety when the rats were exposed to a single, acute, three hours restraint. However, when the rats were subjected to the chronic mixed stress of 48 hours of sleep deprivation both the low and high doses of Vit E exaggerated deficits in spatial memory of the rats. Therefore, although antioxidants may protect the brain from oxidative damage associated with increased metabolism of catecholamines and serotonin, excessive levels of anti-oxidant may also have negative effects on cognitive performance.

In this experiment we adapted rats to an experimental diet, supplemented with antioxidants and then tested their response to a series of stressors. We anticipated that this would allow us to evaluate the effects of specific dietary manipulation on various aspects of stress-induced behavioral changes. However, some of the stressors induced prolonged, or irreversible, changes in the rats which had the potential to confound the response to a subsequent stress. Therefore, future studies will involve adapting different groups of rats to a particular diet and testing the response of each group to a specific stress.

The Effect Of Dietary Amino Acids on Learning And Memory In Acutely Stressed Rats
Gennady Smagin and Igor Rybkin

Relative concentrations of brain neurotransmitters can be altered by availability of their

precursor amino acids. Tryptophan (TRP) enhances the synthesis of serotonin, which may influence learning ability. Histamine is a neurotransmitter with a variety of physiological roles such as thermoregulation, locomotion and neuroendocrine regulation. It has been reported that histamine, injected icv, improved learning and memory in passive and active avoidance tests in rats (De Almeida & Izquiero, 1986; 1988). In addition, the central cholinergic system has been shown to play an important role in learning and memory (Hasselmo & Bower, 1993) and brain histamine modulates acetylcholine release in vivo (Prast et al., 1994). Peripherally administered histamine does not cross the blood-brain barrier in the normal state, and it is necessary to use L-histidine (HIS), the precursor of histamine, to activate central histaminergic systems (Schwartz et al., 1972).

The aim of these experiments was to investigate the involvement of the central histaminergic and serotonergic systems in spatial learning and memory in rats.

Methods and Results

Experiment 1. Male Wistar rats (275 g, n=16) were adapted to consume a liquid diet for 5 days then half of the animals continued to receive the control diet while the others were fed a diet containing 3.5% L-histidine. After 7 days rats were tested for exploratory behavior in the open field and on days 8 and 9 were tested for reference and working spatial memory in the Morris water maze (learning set task paradigm). Rats were sacrificed on day 10. Adrenals and thymus glands were weighed, trunk blood was collected and the brain dissected for neurochemical analyses.

Supplementing the diet with 3.5% histidine did not produced any significant changes in the body weight gain or food intake of the rats. Behavioral tests also did not reveal any effect of diet on exploratory behavior or spatial memory (Figure 2). Neurochemical analyses of different brain regions revealed a significant increase in 5-HIAA concentrations in the hypothalamus of animals fed the histidine diet, none of the other regions measured were affected.

Experiment 2. Twenty-four male Wistar rats (275 g) were adapted to liquid diet. One group of animals received control diet (n=12), and the other group received a diet supplemented with 4.5% of L-histidine (n=12). After 10 days, half of the rats from each dietary group (n=6) were subjected to 3 hours of restraint. Immediately after stress the animals were tested in the Morris water maze Place Set learning Task. Testing was repeated the following day (no stress applied), with the platform in a different position. After behavioral tests rats were maintained on the liquid diets for 5 days, experimental groups were switched, rats that had previously acted as controls were restrained for 3 hours and all of the animals were sacrificed immediately after stress. Trunk blood was collected and brain regions were dissected for neurochemical analyses.

Measuring spatial memory in the Morris water maze did not reveal significant differences on day 1 between the control and HIS groups, but on day 2, in the second trial, animals from the HIS group showed a significant reduction in distance swum to find the platform, implying an

improvement of working memory (Fig 2). Two way ANOVA (set x treatment) showed a significant effect of treatment ($P=0.02$) and set ($P=0.01$). Restraint prior to behavioral testing did not significantly change behavior of rats fed control diet. However rats fed the histidine diet and then restrained traveled an increased distance to find the platform in the second trial (Fig 2). ANOVA showed a significant difference between HIS and HIS+stress groups in trial 2 on days 1 and 2 of testing ($P<0.05$).

Restraint increased serum concentrations of corticosterone in both dietary groups, however, the stress-response of animals fed the histidine diet was significantly lower than in those that were fed control diet (Fig 5). Two way ANOVA showed a significant effect of diet and treatment on plasma corticosterone concentrations.

Analyses of neurotransmitter concentrations in the hippocampus revealed increased serotonergic activity in response to stress, as indicated by an increased concentration of 5-HIAA and 5-HIAA/serotonin ratio, but no effect of diet. Similar changes were observed in the striatum and hypothalamus. Activity of the dopaminergic system was also increased in the hypothalamus and striatum of stressed compared with control rats.

Experiment 3. Twenty-four male Wistar rats (275 g) were adapted to liquid rodent diet for 5 days then one group of 12 animals received control diet, and the other group received a diet with 3% of L-TRP added (n=12). Experimental design was the same as that described for Experiment 2.

There was no effect of diet composition on food intakes or body weights of the rats prior to stress. The results of the Place Learning Set Task are shown in Figure 3, there was no effect of stress or diet on working or spatial memory of the rats. Restraint caused a significant increase in plasma corticosterone in both dietary groups (Figure 3).

Analyses of neurotransmitters in the hippocampus showed an increase in serotonergic activity in response to stress (Increased 5-HIAA/serotonin ratio), but no effect of diet. Similar changes in serotonergic activity were found in the brain stem and hypothalamus. Two way ANOVA showed a significant effect of diet on 5HIAA and TRP concentrations in the hypothalamus (Figure 4). Activity of dopaminergic system was also increased in the hypothalamus, but the differences were not statistically significant.

Conclusions

Based on these results we can conclude, that increasing dietary l-histidine affects spatial memory in rats, as measured in the Morris water maze learning set task paradigm. Whether or not this response involves the interaction of cholinergic and histaminergic systems remains to be determined. Administration of a diet containing excess l-TRP changes the concentrations of TRP and neurotransmitters in hypothalamus, hippocampus and brain stem but had no effect on spatial learning and memory, assessed in the Morris water maze learning set task paradigm.

Restraint caused a significant increase in serum corticosterone and increase serotonin metabolism in a number of brain sites, independent of diet composition. Future studies examining the effect of acute stress on memory will be determined in rats adapted to our new, reduced amino acid diet, described below.

Modulation Of Trypotophan Availability In Sleep Deprived Rats
Bradley Youngblood, Ruth Harris, Gennady Smagin, David Elkins and Jun Zhou

In previous experiments we have demonstrated that 96 hours of rapid eye movement sleep deprivation (REMd) results in a significant impairment of spatial reference memory. Changing the macronutrient content of the diet had no beneficial effects on the cognitive function of the rats nor did it improve any of the physiological responses to REMd, such as weight loss. In all of the studies completed to date we have found that central serotonin metabolism, measured as the 5-HIAA:5-HT ratio, was increased in several brain areas, including the hippocampus, hypothalamus and brain stem. Changes in central serotonin metabolism in response to stress are well documented. central serotonin concentrations and metabolism are stimulated by corticosterone (Jhanwar-Uniyal et al., 1987; Luine et al., 1993) and reduced in adrenalectomized rats (Jhanwar-Uniyal et al., 1987). Luine et al. (1993) also reported that the corticosterone-induced change in serotonin metabolism may be associated with impaired spatial memory, measured in a radial arm maze. Recently , Graeff et al., (1996) have proposed that the increased serotonin metabolism is an adaptive response to stress that attenuates stress.

In the two experiments described here we attempted to modulate central serotonin metabolism by altering availability of the serotonin precursor, tryptophan. In the first experiment we tried to reduce tryptophan availability by feeding a diet containing a high concentration of leucine, an amino acid that competes for tryptophan transport at the blood brain barrier. In the second experiment we attempted to increase tryptophan uptake by increasing dietary tryptophan concentration.

Methods and Results

Experiment 1: Male Wistar rats (300 g) were housed individually and adapted to the liquid control diet for 5 days. The diet contained 14% casein as the protein source. After 5 days on control diet rats were divided into two groups and offered either the control or 3% L-leucine diet for 3 days prior to being placed in experimental conditions. There were 3 treatment groups for each dietary condition: REMd animals housed on small platforms in a water tank. Tank controls (TC), housed on larger platforms in a water tank and cage controls (CC) housed in normal shoe-box cages. During the 96 hour experimental period daily body weights and food intakes were recorded.

On days 2, 3 and 4 of REMd working and reference spatial memory was measured using a Place-Learning Set task (Auer et al., 1989) in a Morris Water Maze, as described previously. The latency and distance swum to a hidden platform was measured from six different starting

points (sets) around the tank with duplicate trails from each starting point. The results were analyzed by plotting latency or distance swum against set for each of the two trails. The area under the curve was calculated as the best representation of performance. On Day 4 rectal temperatures of the rats were measured prior to the rats being tested in the water maze. On that afternoon rats were killed, blood was collected for analysis of corticosterone. Adrenal glands, thymus glands and epididymal fat were weighed. The hypothalamus, brain stem, hippocampus and frontal cortex were dissected for measurement by HPLC of neurotransmitters and their metabolites.

Experiment 2: Experiment 2 was identical to Experiment 1 except that the rats were fed either control diet or a diet containing 3% tryptophan. Rectal temperatures were not recorded on day 4 and only 6 rats per treatment group were tested in the experiment.

Table 4 shows the results for body weights, food intakes, organ weights and serum corticosterone of rats in Experiment 1. There was no effect of diet on weight change, fat pad weight, food intake, rectal temperature or thymus weights of the rats although both TC and REMd rats lost considerable amounts of weight, had elevated body temperatures and had reduced thymus weights compared with cage controls. The L-leucine diet caused a significant increase in serum corticosterone in both the TC and REMd rats but not the cage controls. As corticosterone was already elevated in these animals, it suggests that the L-leucine diet exacerbated the stress response.

Table 4 : Body Weights, organ weights, food intake and body temperatures in Experiment 1.

	Cage Controls		**Tank Controls**		**REMd**	
	Control	Leucine	Control	Leucine	Control	Leucine
Start Weight (g)	339 ± 7	352 ± 8	343 ± 7	352 ± 6	353 ± 6	346 ± 7
Weight Change (g/96 hr)	17 ± 4^{A}	19 ± 4^{a}	-31 ± 4^{B}	-34 ± 6^{b}	-30 ± 3^{B}	-36 ± 4^{b}
Food Int Day 4 (g/96 hr)	84 ± 9	95 ± 7	71 ± 7	67 ± 7	91 ± 4	82 ± 10
Thymus (mg)	564 ± 35^{A}	521 ± 32^{a}	361 ± 19^{B}	371 ± 18^{b}	396 ± 33^{B}	381 ± 34^{b}
Adrenals (mg)	54 ± 3	66 ± 4*	58 ± 3	69 ± 4*	59 ± 4	67 ± 4
Rectal Temp. (°C)	38.1 ± 0.1	37.9 ± 0.1	38.8 ± 0.1	38.7 ± 0.1	38.7 ± 0.2	39.0 ± 0.2
Epididymal fat (g)	4.2 ± 0.4^{A}	4.1 ± 0.3^{a}	3.0 ± 0.2^{B}	3.0 ± 0.2^{b}	3.3 ± 0.3^{B}	3.1 ± 0.4^{b}
Serum Corticosterone (ng/ml)	35 ± 11^{A}	22 ± 3^{a}	170 ± 64^{AB}	234 ± 62^{b}	281 ± 75^{B}	368 ± 49^{b}

Data are means ± sem for groups of 8 rats. All parameters were analyzed by two-way analysis of variance and post-hoc unpaired t-tests. Superscripts indicate a significant difference between groups. An asterisk indicates an effect of diet within treatment group.

Water maze performance of rats in Experiment 1 on days 2 to 4 of REMd is shown in Figure 5. There was a significant effect of treatment on swim speed on all three days of testing, therefore, distance to find the platform was considered the best indicator of spatial memory. In rats fed control diet performance in trial 1, representative of reference memory, there was no effect of treatment until day 4 when REMd rats had a significantly impaired spatial memory compared with either TC or cage control rats. In trial 2, indicative of working memory, there was no significant effect of treatment on any of the 3 days of testing. In rats fed the l-leucine diet there was a significant effect of treatment on reference memory on all three days. On day 2 both TC and REMd rats performed less well than cage controls but on days 3 and 4 only the REMd rats were significantly impaired compared with cage controls. The leucine diet also caused an impairment of working memory in REMd rats compared with both TC and CC rats on Day 4.

In the hypothalamus there was a significant effect of stress on 5-HIAA concentration and 5-HIAA:5-HT ratio, which was increased in TC and REMd rats, but no effect of diet. In the hippocampus similar changes were observed but 5-HT concentration was also decreased by stress, thus exaggerating the effect on serotonin metabolism (see Figure 6). There was no effect of diet or stress on any of the parameters measured in the striatum. In the brain stem norepinephrine concentration was decreased in CC rats fed the leucine diet, compared with those on the control diet, and it was also decreased in REMd rats fed the control diet compared with CC rats fed the same diet. There was no effect of diet on tryptophan concentration in any of the brain areas analyzed.

Body weight, food intakes and organ weights of rats in Experiment 2 are shown in Table 5. The tryptophan diet reduced food intake and suppressed weight gain in control rats. All stressed rats lost similar amounts of weight. Tryptophan also caused a reduction in thymus weight of all treatment groups, suggesting an exaggeration of the response to stress. There were no beneficial effect of tryptophan supplement on working or reference memory of the rats and there was no effect of diet on central concentrations of tryptophan or on serotonin turnover.

Table5: Body Weights, organ weights and food intake in Experiment 2.

	Cage Controls		**Tank Controls**		**REMd**	
	Control	Tryptophan	Control	Tryptophan	Control	Tryptophan
Start Weight (g)	340 ± 4	329 ± 11	331 ± 8	333 ± 7	343± 7	327 ± 7
Weight Change (g/96 hr)	18 ± 2	7 ± 3	-23 ± 7	-31 ± 4	-31 ± 2	-29 ± 2

Food Int Day 4 (g/96 hr)	83 ± 4	73 ± 6	60 ± 13	52 ± 8	57 ± 9	66 ± 5
Thymus (mg)	581 ± 37	567 ± 47	494 ± 58	370 ± 25	445 ± 27	385 ± 24
Adrenals (mg)	58 ± 3	57 ± 2	68 ± 3	66 ± 3	66 ± 5	68 ± 4

Data are means ± sem.

Conclusion

The dietary manipulations in these two experiments did not achieve the changes in serotonin metabolism that we intended. Possibly because the control diet had a high concentration of casein which supplies sufficient tryptophan to maintain serotonin metabolism even in the presence of increased l-leucine concentrations. The results from the leucine experiment suggest that the amino acid imbalance caused by this diet was an independent stressor that exaggerated the impairment of cognitive performance in REMd rats. Therefore, the following experiment was carried out to determine the minimal levels of dietary amino acid required to maintain normal growth in 350 g Wistar rats. Identification of the minimal amino acid requirement would optimize the likelihood of seeing a response to amino acid supplementation in future studies.

Methods and Results

Twenty four male Wistar rats had free access to the 100% diet described below and water. After a week the rats were divided into four weight matched groups and each group was fed one of the four diets described below. The 100% diet contained 100% of the amino acids required by young growing rats (Gahl et al., J, Nutr. 121, 1991) the other diets contained progressively decreasing proportions of this requirement but the relative ratios of the amino acids were maintained.

Rats fed the 40% amino acid diet gained significantly less weight than those fed the 100% diet (see Figure 7). Repeated measures analysis of variance indicated that weight gain of rats fed the 60% diet was not significantly different from that of rats fed the 100% diet. As dietary amino acid concentration decreased daily food intake increased slightly, but not enough to make amino acid intake equal between groups (see Figure 7).

Diet Composition (g/Kg)

	100%	**80%**	**60%**	**40%**
Amino acid mix	121	96.8	72.6	48.4
Corn oil	100	100	100	100
Alphacel	30	30	30	30
Sucrose	140.8	145.6	150.5	155.3
Starch	563.2	582.6	601.9	621.3

AIN 76 Vitamin mix	10	10	10	10
AIN 76 Mineral mix	35	35	35	35

Amino Acid Content Of 60% Diet

Amino Acid	**g/Kg**
Arginine	3.6
Histidine	1.8
Isoleucine	3.0
Leucine	4.5
Lysine	4.2
Methionine	2.4
Cystine	1.2
Phenylalanine	3.18
Tyrosine	1.62
Threonine	3.0
Tryptophan	0.9
Valine	3.6
Alanine	2.4
Aspartic acid	2.4
Glutamic acid	24.0
Glycine	3.6
Proline	2.4
Serine	2.4
Asparagine	2.4
Total	**72.6**

Conclusions

The results from this experiment indicate that a diet containing 60 % of the NRC requirements for young, growing animals is adequate to support normal growth in adult male rats. Research Diets have formulated a liquid diet that maintains the amino acid : energy ratio of our 60% diet and we will be comparing the liquid and powder diets directly to ensure equality. In future we intend to use the liquid diet as the control diet for stress studies as the reduced amino acid content will optimize the opportunity to observe effects of amino acid supplementation on behavior while feeding a diet that is adequate for normal growth and maintenance.

The Effect of Restraint Stress on Food Intake and Body Weight

Igor Rybkin

In a previous study we found that a single 3 hour restraint caused a significant reduction in food intake during the next 24 hours. Measurement of intake during 4 to 5 hour intervals during these 24 hours revealed that all of the change in food intake could be accounted for during the first few hours of the dark period when rats eat a large portion of their daily intake. The objective of this experiment was to determine whether there was a difference in response of rats subjected to restraint at either the beginning or at the end of the light period. In addition, measurements of hypothalamic neurotransmitter concentrations and peripheral hormone concentration provided an indication of some of the mechanisms that may be involved in the response.

Methods and Results

Adult , male Sprague- Dawley rats (n=64), 360-380 g, were individually housed with free access to standard rat chow and tap water.

Design of experiment: Due to the large number of animals involved, the study was divided into two experiments. Both experiments had a same general design but differed in the time of day that stress was applied. Rats were adapted to cages and handling for one week, they were then monitored for seven days before stress (baseline), and for 9-10 days after stress (recovery period). Body weights and food intakes were measured daily at 8:00 am in both experiments. On the day of stress rats were divided into two groups (control and restraint) matched for average body weight.

Experiment 1: On the day of stress the rats were moved from their home cages to another room at 16:00 p.m. Sixteen control rats were placed in regular shoe-box cages without any food or water, and 16 stressed rats were placed in perspex restraining tubes. After 3 hours all animals were returned to their home cages with free access to food and water. Food and water intake were recorded: 08:00-16:00 - before stress; (16:00-19:00 stress- no data); then 19:00-21:00; 21:00-23:00; 23:00-1:00; 1:00-4:00; 4:00-8:00. After a recovery period of 10 days following stress the control and stressed groups were switched and were either restrained for 3 hours or placed in control cages in the same conditions as used previously. All of the rats were killed at the beginning of the dark cycle between 19:40 and 20:15 hours.

Experiment 2: This experiment was identical to experiment 1 except that the rats were stressed in the morning from 8:00 to 11:00 a.m. Food and water intake were recorded: (08:00-11:00 - stress - no data); then 11:00-15:00; 15:00-19:00; 19:00-21:00; 21:00-23:00; 23:00-1:00; 1:00-4:00; 4:00-8:00. The recovery period for this experiment was same as in experiment 1 and after the second stress animals were decapitated at the beginning of the dark cycle between 19:40 and 20:15 hours.

Results - Experiments 1 and 2. In experiment 2 stressing the rats early in the morning appeared to have a greater effect on body weight than did stress at the end of the light period. Both body weights and weight change from baseline were significantly different between control and restrained rats on all of the days of recovery. In both experiment 1 and experiment 2 there was a significant effect of restraint on 24 hour food intake following restraint (see Figure 8). There was no acute effect of stress on water intake of the rats and no effect of stress on water intake during the recovery period of experiment 1 or experiment 2. Measurements of food intake at intervals during the 24 hours immediately following restraint stress in the two experiments are shown in Figure 9. In experiment 1, when rats were restrained at the end of the light period, food intake was significantly reduced in stressed rats, compared with controls, only during the first 2 hours (19:00 - 21:00 hours) of the dark period. This difference accounted for the difference in 24 hour intake of the 2 groups. In experiment 2, when rats were restrained at the start of the light period, food intake was significantly reduced in stressed rats during the 4 hours immediately following restraint (11:00 - 15: 00 hours), during the first 2 hours of the dark period (19:00 - 21:00 hours) and during the last 4 hours of the dark period (04:00 - 08:00 hours).

Serum glucose, insulin and corticosterone concentrations were measured at the end of the experiment. Two-way analysis of variance indicated that corticosterone was significantly higher in animals killed in experiment 2 than in experiment 1 but that there was no effect of stress. Similarly, serum insulin was significantly higher in both control and restrained rats from experiment 2 than from experiment 1, but there was no effect of stress. The only difference in serum glucose concentrations was an elevation in the stressed rats from experiment 1, compared with their controls. There were no differences in hypothalamic NPY mRNA expression but tissue NPY protein, measured by radioimmunoassay of punches obtained from the paraventricular nucleus indicated a significant elevation of NPY in the stressed rats in experiment 2, compared with their controls. There was no difference in protein concentrations between controls and restrained rats in experiment 1 (see Figure 9).

Experiment 3. In the previous experiments 3 hours restraint stress applied at different times of the day, namely at the beginning and at the end of the light cycle, had different effects on the food intake and body weight loss of rats. Analysis of the circulating hormones, corticosterone and insulin, and the concentration of hypothalamic neuropeptide Y or its expression in the hypothalamus of rats of this experiment did not reveal a clear correlation of these proteins with the physiological responses of the rats. It has been demonstrated that restraint stress increases the activity of the monoaminergic system of the brain (Kennet et al., 1981, Shimizu et al., 1992) and this system has been associated with stress - induced anorexia. Therefore, the objective of this experiment was to determine whether there was any correlation between the time of day that stress was applied and brain monoamines concentration at the start of the dark period, when suppression of food intake is maximal. The activity of the sympathetic nervous system, an important link in the energy expenditure and body weight regulation system, was also measured. Experimental design was the same as that used in previous experiments in order to allow comparison of results.

Materials and Methods

Adult, male Sprague- Dawley rats (n=24), weighing 380-400 g, maintained on a standard rat chow and tap water *ad libitum*. Before the start of the experiment the rats were adapted to home cages and handling for one week. The experiment was divided into two periods: baseline (7 days) and day of restraint stress. During the baseline period food intake, water intake, and body weight were measured daily at 8:00 am. Rats were divided into control and restraint groups (n = 6 rats each) matched for average body weight on the day of stress. Two groups (control2 and restraint2) were stressed at the beginning of the light cycle and two groups (control1 and restraint1) were stressed at the end of light cycle.

On the day of stress the rats of control1 and restraint1 groups were moved from their home cages to another room at 7:00 a.m. Six control rats were placed in regular shoe-box cages without any food or water, and 6 stressed rats were placed in perspex restraining tubes (Plas Labs, Lansing, MI). After 3 hours all animals were returned to their home cages with free access to food and water and remained undisturbed until 18:30. The rats of the restraint2 groups were stressed in the afternoon from 15:00 to 18:00 hours. All rats were sacrificed between 19:40 and 20:15 hours by decapitation. Blood was collected for measurement of serum glucose, insulin and corticosterone. Cerebral cortex, hypothalamus and brain stem were analyzed for monoamine content by HPLC and brown adipose tissue expression of uncoupling protein (UCP) mRNA was measured by Northern blot analysis. Data are presented as mean $\pm$ SE of the values obtained from the analysis of data in 6 rats/group. Results from measurements of neurotransmitters and mRNA expression were compared by one-way ANOVA.

Results

The results of HPLC measurements showed that dopamine turnover (DOPAC/DA ratio) in rats stressed in the evening was significantly lower (18%) in the brain stem compared with that in their controls, due to an increased DA content. In contrast, increased (28%) DA turnover in the hypothalamus of the same animals was due to an increased amount of DA metabolite, DOPAC. There were non- significant elevations of NE (2.18 ± 0.11 *vs.* 1.87 ± 0.06) and 5HIAA ($0.763\pm0.0.049$ *vs.* 0.667 ± 0.025) but not of 5-HT in the hypothalamuses of rats restrained in evening compared with their controls. Expression of UCP mRNA in the brown fat pads was not different either in the rats restrained in the morning or in the rats restrained before the start of dark cycle. Serum corticosterone concentration was significantly different between rats stressed in the morning and their controls. Insulin was significantly higher in rats stressed in the afternoon than those stressed in the morning.

Conclusion

The changes in food intake and body weight of rats exposed to restraint at different times of day suggest that stress applied during the early part of the light period has a greater impact than that applied at the end of the light period. There was no difference in expression of NPY

mRNA, indicating that protein synthesis was not changed by stress. However, NPY protein in the PVN of the hypothalamus was significantly elevated in restrained rats in Experiment 2, compared with all other groups. The rats in experiment 2 were restrained at the start of the light cycle, approximately 9 hours prior to being killed, and demonstrated a greater stress-related inhibition of feeding than those stressed late in the light period. Although NPY would be expected to stimulate feeding in these animals it is possible that the high protein concentration reflects a failure to secrete the protein from the cells. Further experiments would be required to fully elucidate the role of NPY in the stress-induced feeding response.

Other investigators have implicated increased hypothalamic serotonin metabolism in the aphagic response to acute stress. Although the difference between monoamine concentration in the hypothalamus and brain stem of the controls and stressed rats was not large, the fact that rats were decapitated an average of 3 hours and 50 min after the beginning of stress should be taken in account. Shimizu et al. demonstrated that the level of hypothalamic 5-HT increased to a maximum 40 min after initiation of immobilization but then began to decrease toward the pre-immobilization levels even while the stress still continued. At the same time, 5-HIAA concentration gradually increased over the whole immobilization period. Levels of NE, DA, 5-HT and their metabolites in our experiment suggested that we caught the tail end of the stress-induced activation of these systems in the rats stressed before the start of the dark cycle. These experiments have been described in a manuscript submitted to American Journal of Physiology (Rybkin et al.) which has been accepted with revision.

B. Progress on Ongoing Projects

Stress and Appetitive Behavior
Bradley Youngblood and Ruth Harris

Operant schedule-controlled behavior has been used to evaluate a the effects variety of drugs, brain lesions and thermal stressors (Ahlers and Salander, 1993; Ahlers et al., 1992; Gauvin et al., 1994; Cancela et al., 1996). Rats have to be food restricted prior to being trained to lever press to receive food pellets. There is no information available on the effects of acute stress on appetitive operant behavior in rats or on whether stress inhibits food intake of restricted rats, however, it is well documented that stress suppresses food intake of ad libitum fed rats (Michajlovski et al., 1988). The studies described here used a small fixed-ratio (FR15 or FR5) schedule of reinforcement for rats, eliminating post-reinforcement pausing, resulting in a maximal rate of rapid responding with frequent reinforcements. We determined the effect of acute restraint stress or restraint stress combined with partial water immersion on performance of the rats in the operant task and on free-feeding behavior.

Experiment 1 - Methods and Results. Twelve male Sprague Dawley rats (325- 400 g: Harlan Sprague Dawley) were food restricted to 85% of control body weight by feeding 12 g chow/day. Six rodent operant chambers were located in a sound attenuated room with the lights out. Each chamber was fitted with a single operant lever, stimulus lamp and pellet dispenser.

All behavioral/experimental contingencies and data collection were accomplished by a computer interfaced by an integrator (Lafayette Instruments) to the operant chambers using Schedule Manager for Windows (Med Associates Inc., St. Albans, VT). All rats were shaped by the method of successive approximations to press the lever for 45 mg food pellets during a 3-cycle training session. All sessions began with a 10-minute time-out period (no reinforcement and stimulus light off). Each time-out period was followed by a 10 minute time-in period (schedule-controlled food reinforcement and stimulus light on). Over successive training sessions, the number of lever-press responses required to produce a food pellet was raised from the initial FR1 to FR15. Training continued until the total number of food pellets earned during all 3 time-in periods for 3 consecutive days varied less than 10%. The total number of food pellets earned were converted to grams by multiplying by 45 mg. All operant sessions were conducted between 1300 to 1500 hr, 5 days per week (M-F). There was one week period of operant recovery training between each experiment described below.

The effect of restraint on free-feeding of rats. Rats were taken from their home cages to another room and placed in restraining tubes for 3 hours. Control rats were placed in shoe-box cages without food or water in the same room as the restrained rats. At the end of the restraint period the rats were placed in the operant chambers and tested for free-feeding food intake by offering preweighed food pots containing 45 mg food pellets. The jars were offered in a 3 cycle 10 minute jar-in/jar-out session, identical to that used for the operant session.

The rats ate significantly more during the free-feeding test in the operant chambers than when they had to lever press for the food. There was no significant effect of restraint on food intake of food restricted rats (see Figure 10).

The effect of restraint stress on appetitive behavior. Only 8 of the 12 rats were included in this experiment as the other 4 did not meet criteria performance. All of the rats were subjected to 3 hours of restraint stress, as described above. At the end of restraint they were placed in the operant chambers and appetitive behavior was measured during 3 cycles of 10 minute time-in/time-out sessions. As shown in Figure 10, there was no effect of restraint on appetitive behavior of the rats, measured as total amount of food acquired during the time-in sessions.

The effect of restraint stress with partial water immersion on free-feeding and appetitive behavior. Eight rats that met criteria performance were included in this experiment. The restraint/water immersion stress involved placing the rats in restraining tubes and then partially immersing the tubes, up to the scapula of the rat, in 25°C water for 3 hours. At the end of stress the rats were tested for free-feeding or appetitive behavior in the operant chambers. The study was of cross-over design so that each animal acted as its own control. As shown in Figure 10, the combined restraint/water immersion stress totally abolished appetitive behavior. During the free-feeding tests the animal ate approximately 50% of their operant intake measured on the day immediately prior to the stress day.

Experiment 2. In the previous experiment RWI suppressed both appetitive and voluntary

food intake of rats, suggesting that the reduced operant behavior was due to a combination of stress-induced effects that included a motor impairment and a loss of appetite. In this experiment the duration of RWI stress required to suppress operant behavior without disrupting free-feeding behavior was determined and then the effect of pre-treating the rats with L-tyrosine was investigated, assuming that this would supplement the catecholamine system of the rats and prevent any stress-associated disruption of performance.

Methods and Results

Sixteen male Sprague Dawley rats were food restricted to 85% of their normal body weight and trained to an FR-5 schedule of food reinforcement in a 3 cycle 10 minute-in, 10 minute-out paradigm. Once the rats met criteria for stable response, 8 were exposed to 30 minutes of RWI prior to placement in the operant chambers. Food intake during the FR-5 schedule was recorded. After one week of recovery the rats were stressed a second time and voluntary food intake measured during 3 10 minute food-pot in, food-pot out schedule was recorded. This stress caused a significant reduction in free feeding behavior in addition to reducing food pellets earned during the FR-5 reinforcement schedule (see Figure 11). After a week of recovery the experiment was repeated but the duration of RWI stress was reduced to 15 minutes. This stress had no significant effect on free-feeding but caused a significant reduction in food reinforcers earned during the first two 10 minute cycles of the reinforcement schedule and significantly reduced response rate during the 30 minutes of positive reinforcement (see Figure 12).

After the rats had been allowed to recover for one week the experiment was repeated except that stressed and control rats were subdivided into 2 groups: one group received an i.p. injection of saline and the other received an i.p. injection of 100 mg/Kg L-tyrosine 30 minutes prior to the start of 15 minutes of RWI stress. There was no difference in operant response of the 2 groups of control rats but L-tyrosine caused a significant reduction in free feeding during the second 10 minute cycle of the reinforcement schedule. The RWI-saline rats reduced reinforcers earned during cycle 1 and free feeding was decreased during cycle 2. The RWI-tyrosine group significantly decreased reinforcers earned and response rate during cycle 3 and the total 30 minute reinforcement period. Free feeding was significantly reduced during the first two cycles of the schedule.

Once the rats had recovered from this stress they were again pre-treated with saline or L-tyrosine and killed immediately after the end of stress. Brain areas were collected **and frozen for** measurement of catechoalmine concentration. These measurements have not been completed as the HPLC is being repaired.

Conclusions

Pre-treatment of the rats with L-tyrosine did not prevent the stress-induced suppression of operant behavior. Injecting the rats with either saline or tyrosine caused a significant reduction in

free-feeding behavior suggesting that the single injection is stressful to the rats. Future experiments will determine whether treating the rats with tyrosine after RWI but before being placed in the operant cages modulates stress-induced changes in operant performance.

Chronic Disruption of Energy Balance in Rats Subjected to Repeated Restraint Stress
Ruth Harris, Bradley Youngblood, Jun Zhou, Shiela Moore, David Elkins, David Horohov and Gennady Smagin

There is conflicting information of the effects of stress on food intake and body weight. Very mild stress of tail pinch increases food intake (Levine and Morley, 1981) whereas more extreme stress, such as restraint or immobilization (Marti et al., 1994), reduces both food intake and consumption of sweet solutions. Repeated immobilization has been reported to suppress food intake for 14 days (Marti et al., 1994), to inhibit release of growth hormone and thyroid stimulating hormone (Armario et al., 1993) and to accentuate the diurnal rhythms of pituitary hormones and of corticosterone release (Marti et al., 1993).

In these experiments we examined the effects of 3 hours of restraint on each of 3 consecutive days on the food intake, body weight and immune functions of rats and the interactions between stress and dietary fat content.

Experiment 1. Twelve male Sprague Dawley rats were adapted to measurements of food intake and body weight for 10 days and then were divided into two groups matched for equal body weight. On the days of restraint the rats were moved to another room. One group was placed in restraining tubes and the controls were put into shoe-box cages without food or water. At the end of restraint they were returned to their home cages. Restraint was repeated on each of 3 successive days from 7.00 am to 10.00 a.m. After the end of stress daily body weights and food intakes were recorded for a further 42 days.

Restraint stress caused a significant inhibition of food intake which returned to control levels by day 7, 4 days after the end of stress (see Figure 13). However, there was no rebound to compensate for the stress-induced hypophagia. Therefore, cumulative food intake measured over 42 days was significantly different between the groups. Restraint caused a significant weight loss, as shown in Figure 13, and body weights of the restrained and control rats were significantly different up to day 42.

Experiment 2. The objective of this study was to expose young 30 day old male rats to a similar stress, three consecutive days of three hours of restraint stress, and determine whether the response was the same as in adult rats in our previous experiments.

Sixteen young (100g) male Sprague Dawley rats were divided into 2 groups of 8, either in a control group or restraint group. The stressed rats were exposed to 3 hours of restraint for 3 days. Body composition , thymus and adrenal gland weights, and serum corticosterone were measured after eight days of post-stress recovery.

Beginning on day 2 of stress, food intake of the restrained group was significantly decreased compared to controls (df 1, 14 ; F 4.43 ; $p < 0.053$, day 2: df 1, 14 ; F 13.96 ; $p < 0.002$, day 3). Food intake remained significantly decreased only on post-stress recovery day 1 (df 1, 14 ; F 9.51; $p < 0.008$). Body weights of the groups were not significantly different on any day of the experiment. Body composition, thymus and adrenal weights, and serum corticosterone of the groups were not significantly different after 8 days of post-stress recovery (see Table 6).

Table 6: Body Composition of Young Rats Exposed to Repeated Restraint

	Control	Restrained
n	8	8
Carcass Weight (g)	168 ± 3	169 ± 2
Carcass Fat (g)	11.1 ± 0.4	10.8 ± 0.4
Carcass protein (g)	31.9 ± 0.6	33.0 ± 0.3
Carcass Water (g)	119.7 ± 2.4	119.8 ± 1.6
Carcass Ash (g)	5.1 ± 0.2	5.3 ± 0.2
Thymus (mg)	719 ± 32	655 ± 20
Adrenals (mg)	32 ± 1	32 ± 1
Corticosterone (ng/ml)	21 ± 7	19 ± 7

Data are means ± sem for groups of 8 young male Sprague Dawley rats that were 30 days old when they were exposed to repeated restraint. Body composition was determined 8 days after the end of restraint when the rats were 41 days old. There were no significant effects of repeated restraint on any of the parameters measured at the end of the experiment.

Experiment 3. The objective of this experiment was to determine whether feeding the rats a high fat diet, which induces weight gain in ad libitum fed rats, would prevent the weight loss seen in Experiment 1.

Methods and Results

Thirty two adult male Sprague Dawley rats were weighed daily and after 6 days were divided into two groups, matched for average weight. One group was fed chow (low fat diet ~10% kcal fat) and the other group were fed a high fat diet (80% chow, 20% Crisco~ 40% kcal fat). After 9 days on the diet each group was subdivided into two more groups, matched for weight, within each dietary treatment. One subgroup was subjected to 3 hours of restraint from 9.00 a.m to 12.00 p.m on three consecutive days. The rats were decapitated on Day 5 after the end of stress. Blood was collected for glucose, insulin and corticosterone analysis. Epididymal fat was weighed and snap frozen for determination of leptin mRNA expression. Adrenal glands, thymus and carcass weights were recorded. The hypothalamus was dissected and frozen for HPLC determination of catcholamines. Carcasses were frozen for determination of body composition.

Spleens from 4 animals per treatment group were pooled and used for an in vitro spleen lymphocyte proliferation assay. Interleuken-2 receptor (IL-2R) expression on lymphocytes was assessed during the first three days of culture using fluorescently labeled human IL-2 and FACS analysis.

Rats fed the high fat diet gained more weight than those on the low fat diet. Both high-fat and low-fat fed rats lost weight in response to restraint (see Figure 14). Animals fed the high fat diet ate less food than those on powdered chow and restraint caused a significant reduction in food intake of both dietary groups that did not return to control levels until 4 days after the end of restraint, day 14. Cumulative food intake during the 8 days from the start of stress to the end of the experiment was significantly reduced in rats fed a high fat diet and restraint caused a significant reduction in intake for both dietary groups.

Table 7 shows the serum hormone concentration and body composition of the rats, measured on Day 5 after the end of stress. There was no effect of diet or stress on adrenal weight, serum corticosterone or insulin concentrations. Stress significantly reduced serum glucose. Both groups of restrained rats weighed significantly less than controls at the end of the study. Carcass analysis revealed that the weight loss in low-fat fed rats was due to loss of both lean and fat tissue. In contrast, the weight loss in high-fat fed rats was totally accounted for by loss of lean tissue. Measurements of hypothalamic catecholamine concentrations by HPLC analysis did not show any significant effect of diet or treatment.

The results of the spleen lymphocyte assays are shown in Figure 15. In rats fed the low fat diet there was little effect of stress on cell proliferation in response to PWM, although there was a tendency for less proliferation at the highest and lowest doses used. In contrast, in rats fed the high fat diet restraint caused a substantial inhibition of lymphocyte proliferation at all doses of PWM tested. This diet/stress interaction was also apparent in the proliferative response of the cells to IL-2 as cells from stressed, high-fat fed rats failed to respond to the cytokine. Despite the decreased proliferative response to IL-2, cells from stressed, high-fat fed rats had a somewhat increased level of IL-2R expression, compared with controls (see Figure 15).

Table 7: Serum hormones and carcass composition of restrained rats fed low and high fat diets.

	LF Control	LF Rest	HF Control	HF Rest
Corticosterone (ng/ml)	16 ± 2	28 ± 5#	45 ± 21	25 ± 5*
Glucose (mg/dL)	30 ± 1	28 ± 1#	32 ± 1	29 ± 1*
Insulin (ng/ml)	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.1
Carcass Composition (g/rat)				
Weight	384 ± 5	370 ± 4#	403 ± 7	383 ± 5*
Water	253 ± 3	246 ± 4	260 ± 5	246 ± 4*

Protein	88 ± 2	86 ± 1	96 ± 1	91 ± 2
Fat	29 ± 2	24 ± 2#	34 ± 3	32 ± 2
Ash	14 ± 1	14 ± 1	13 ± 1	14 ± 1
Adrenals (mg)	50 ± 4	52 ± 6	54 ± 4	51 ± 6
Thymus (mg)	294 ± 59	266 ± 52	335 ± 60	270 ± 56*

Data are means ± sem for groups of 8 rats killed 5 days after the end of restraint stress. An asterisk indicates a significant difference between HF controls and HF restrained rats. # indicates a significant difference between LF controls and LF restrained rats. Statistical significance was determined by two-way analysis of variance and post-hoc t-test.

Experiment 4. In the previous experiment a high fat diet was made by combining vegetable shortening with chow, lowering the proportion of calories provided by protein and the micronutrient concentration of the diet. This experiment was carried out to determine whether the failure to gain lean body mass in stressed rats fed high fat diet was because the percent calories from protein were limiting in the high fat diet.

Methods and Results

Ten male Sprague Dawley rats were offered a high fat diet described in Table 8 which provided 16% kcal from protein and 40% kcal from fat, a combination of corn and coconut oil. Food intakes and body weights were recorded daily and 9 days later the rats were divided into 2 weight matched groups. One group was exposed to repeated restraint and one group was control. Five days after the end of restraint the rats were killed for determination of body composition.

Table 8: Diet Composition

	High Fat Diet
Casein	200
Vitamin Mix AIN 76	10
Mineral Mix AIN 76	34
Corn Oil	125
Coconut oil	100
Starch	243.5
Sucrose	243.5
Alphacel	40
DL-Methionine	4
% kcal fat	40.8
% kcal protein	16

As in the previous experiment exposure to repeated restraint caused a reduction in food intake and body weight, however the differences between control and stressed rats were not

statistically significant. Body composition of the rats is shown in Table 9. Although there was no difference between carcass weights of the two groups of rats at the end of the period of restraint, the stressed rats had a higher carcass fat content and a lower lean body mass than the controls.

Table 9: Body Composition Of Adult rats exposed to repeated restraint

	Control	Restrained
Corticosterone ng/ml	20 ± 3	26 ± 3
Adrenals mg	58 ± 2	58 ± 1
Thymus mg	485 ± 25	466 ± 52
Carcass Composition (g/rat)		
Weight	406 ± 12	395 ± 7
Fat	51 ± 8	58 ± 7
Water + Protein	345 ± 7	323 ± 9*
Ash	10 ± 1	13 ± 1

Data are means ± sem for groups of 5 rats.

Conclusions

These results show that exposing rats to repeated restraint causes a prolonged reduction in food intake and body weight. Feeding the rats a high-fat diet does not prevent weight loss although the diet caused the rats to gain more weight than on the low fat diet. Although this is not a large weight loss, the indication that all of the loss is associated with lean mass in rats fed a high fat diet, suggests that it could have a significant impact on physical performance of individuals operating in a highly stressful environment. It should be noted that the high-fat diet used in this experiment was within the range of fat intake of military personnel. The diet/stress interaction, apparent in impairment of in vitro immunological measures, suggests that the combination of a high fat diet and stress cause a substantial shift in regulation of immune function. Further studies are needed to determine whether these changes represent a decreased resistance to pathogens. In contrast to the observations in adult rats, exposure of young male rats to repeated restraint briefly suppressed their food intake but body weight and body composition were unaffected.

The specific loss of lean body mass in rats fed a high fat diet and exposed to repeated restraint is not due to an inadequate amount of protein in the diet. Future experiments will investigate the changes in peripheral metabolism responsible for the specific loss of lean tissue in restrained rats and the role of fatty acids in mediating changes in immune function.

Validation of Use of Antisense Oligonucleotides for Investigation of Mechanisms Mediating Behavioral Responses

Gennady Smagin

Recently, antisense oligonucleotides have been successfully used in various in vivo and in vitro systems to selectively arrest the translation of target mRNA into functional proteins. The use of these antisense knockdown strategies provides novel information concerning the function of a given protein, by examining the results of decreased production of the particular protein.

Experiment 1. This experiment validates the use of CRF antisense oligonucleotide for stress studies. A CRF antisense oligonucleotide (ON) was designed based on the sequence of CRH mRNA (5' AGC CGC ATG TTT AGG GGC 3') that corresponds to the initiation codon of CRF mRNA. The complementary sense ON and aCSF served as controls. Injections of CRF antisense or sense ON into the lateral ventricle were made using a microinfusion pump with a Hamilton syringe connected to a 26 gauge injection needle via polyethylene tubing. Animals were injected with 30 mg of ON 3 times at 12 h intervals, with the last injection 6 hours before the experiment. Two groups of animals were used: CRF sense (n=3) and CRF antisense (n=3). On the day of experiment, animals were tested in the open field (5 min session), where the following behaviors were scored: total locomotor activity and time spent in the initial quadrant. After behavioral tests, animals were immobilized for 15 min in commercial plastic restrainers and blood samples were taken every 15 minutes for 2 hours for measurement of circulating corticosterone. Figure 16 represents the results of this pilot experiment. Immobilization for 15 minutes produced an increase in plasma corticosterone concentrations with the peak at 30 min after the onset of immobilization. Animals that received icv injections of CRF mRNA antisense ON displayed an attenuated response of HPA axis to stress. Corticosterone measured 45 min after the onset of stress was significantly different between the two groups. Two way ANOVA (treatment and time) revealed a significant difference between groups (P=0.009).

Analysis of behavior in the open field reveals that animals injected with antisense CRF ON spent less time in the initial quadrant of the open field, and traveled in the open field considerably more compared to sense ON injected controls (Figure 16). A decrease in the time spent in the initial corner of the open field is indicative of decreased anxiety, however, due to the small number of animals per group, there was no significant difference between groups. These results are similar to those obtained with the use of CRF antagonist, ahCRF. Animals injected with ahCRF icv increase locomotor activity.

Experiment 2. Considerable evidence suggests a role for endogenous brain corticotropin releasing factor (CRF) systems in appetite regulation, energy balance and etiology of eating disorders. Food intake is diminished by administration of CRF or urocortin and treatments that increase endogenous hypothalamic CRF production, such as stress, tumor induction or appetite-suppressing drugs (Appel et al., 1991; Krahn et al., 1986; Spina et al., 1996).

Recently, receptors for CRF have been cloned and functionally expressed. A CRF_1

receptor exists in CRF_{1a} and CRF_{1b} forms (Chen et al., 1993; Perrin et al., 1993). A CRF_2 receptor also exists in two forms, tentatively named 2a and 2b (Lovenberg et al., 1995). The receptors have different pharmacological profiles with respect to the CRF-related peptides and show heterogeneous tissue distribution. CRF_2 receptor mRNA expression was undetectable in neocortex or cerebral cortex, in contrast with CRF_1 receptor expression in these regions (Potter et al., 1994). Similarly, CRF_2 receptor expression was unremarkable in pituitary lobes where CRF_1 receptor expression was detectable. This heterogeneous distribution of CRF receptor subtypes is likely to reflect distinct functional roles for CRF_2 and for CRF_1 receptors. Owens et al. (1995), using antisense ON directed against the CRF_1 receptor, found a significant attenuation of the ACTH response following a 30-min challenge with CRF in rat pituitary cultures. In this experiment we determined whether specific suppression of CRF1 and CRF2 receptors had differential effects on CRF and urocortin (UCN) induced hypophagia and activation of the HPA axis.

Male Sprague Dawley rats (275-300 g) were implanted with a single 21 gauge cannula aimed in the lateral cerebral ventricle and were allowed to recover for 7 days. A CRF_2 receptor antisense ON was designed based on the sequence of CRF_2 receptor mRNA, (5' CGC GTC CAT TGC GCT CCG GAG 3') that corresponds to the region upstream and downstream of the initiation codon of CRF_2 receptor mRNA. The CRF_1 receptor antisense ON was based on the published sequence of CRF_1 receptor mRNA (5' GCC GTC CCA TCC TCG GGC TCG 3'). The complementary sense ON and saline served as controls. CRF_1 and CRF_2 antisense and sense oligos were injected 3 times, every 12 hours icv (20 ug per injection, with the last injection 3 hours before the experiment). Animals were food deprived for 24 hours. On the day of the experiment, animals received an injection of CRF icv (3 ug) and food intake was measured and blood samples were collected every 30 min during 2.5 hours. Six groups were formed: saline/saline, saline/CRF; sense ON/saline, sense ON/CRF; antisense ON/saline, antisense ON/CRF.

After the experiment with CRF, sense and antisense ON treatment was continued for two more days (four injections), experimental groups were reversed and animals were injected with urocortin (3 ug icv). Six groups were formed: saline/saline, saline/urocortin; sense ON/saline, sense ON/urocortin; antisense ON/saline, antisense ON/urocortin.

Animals that received injections of antisense and sense oligo to CRF_1 receptor mRNA significantly decreased their food intake in response to CRF injection (Fig 19). However, the 24 h food intake was not affected. Treatment with antisense and sense ON to CRF_1 receptor mRNA did not affect the HPA response to CRF infusion, all animals injected with CRF display an increase in serum corticosterone concentration which peaked at 90 min after injection (Fig 19). Control animals displayed a slightly elevated serum corticosterone level.

Infusion of CRF in animals treated with saline and sense ON to CRF_2 receptor mRNA caused a decrease in food intake and increased concentrations of corticosterone (Fig 20). Food intake was not different from the control group (sense ON/saline) in animals treated with

antisense oligo to CRF_2 receptor mRNA and antisense ON significantly attenuated the HPA response to CRF infusion.

Urocortin, a specific CRF_2 receptor agonist, injected icv in the animals treated with saline, sense and antisense ON to CRF_1 receptor mRNA produced a significant decrease in food intake. Treatment with antisense ON to CRF_2 receptor mRNA significantly attenuated the effect of urocortin on food intake (Fig 21).

Conclusion

The results of this study confirm previously published data (Skutella et al., 1994) that antisense ON can be used to investigate the biological effects of peptides and particularly, CRF. Attenuation of behavioral and neuroendocrine response to CRF and CRF_2 agonist, urocortin, by antisense ON to CRF_2 receptor mRNA suggests the involvement of this type of receptor in the regulation of food intake and activation of the HPA axis. Experiments in progress will determine the effect of treatment with oligonucleotides on CRF receptor binding in the rat brain.

Future studies will take advantage of the antisense technology to determine the roles of CRF, UCN, CRF1 receptors and CRF2 receptors in specific behaviors initiated by stress.

Neurochemical Properties Of Urocortin, A Novel CRF-Related Peptide
Gennady Smagin

A key neurohormone that plays a significant role in integrating the response to stress throughout the neuro-endocrine-immune axis is corticotrophin-releasing factor (CRF). CRF regulates glucocorticoid responses to stress and mimics most of the behavioral responses to stress. Until recently, only one endogenous CRF had been isolated from the mammalian brain, suggesting that only CRF was directly involved in stress-induced behavioral changes. However, the identification of another neuropeptide of the CRF family, Urocortin (UCN), suggested a potential physiological role for endogenous UCN in activating central CRF receptors. Published data led to the hypothesis that central infusion of UCN may produce behavioral and neurochemical effects that only partially overlap with those produced by CRF.

Experiments were performed to identify the neurochemical changes produced by icv infusion of UCN.

Experiment 1: The effect of icv infusion of UCN on catecholamine and indolamine metabolism in the brain. Male Sprague-Dawley rats (275-320 g) were implanted with the stainless steel cannulae aimed into the lateral cerebral ventricle. After 7 days of recovery, 3 ug of UCN (in 3 ul of aCSF) was injected icv over a 2 min interval, control animals received injections of aCSF alone. Animals were decapitated 1 and 2 hours after injection and trunk blood was collected. Brains were removed, hypothalamus, brain stem and the medial prefrontal cortex were immediately dissected and snap frozen. Tissue samples were extracted using 0.1M perchloric

acid, and catecholamines, indolamines and metabolites were assayed using HPLC with electrochemical detector. Serum corticosterone was assayed by RIA .

Administration of 3 ug of UCN icv produced a significant increase in serum corticosterone 1 and 2 hours after the infusion, see Figure 22, consistent with previously published data that UCN activates the HPA. UCN infusion also affected central noradrenergic, serotonergic and dopaminergic systems. MHPG/NE ratio was significantly increased in the medial prefrontal cortex (PFM) 1 and 2 hours after infusion. 5-HIAA/5-HT ratio, an indicator of activation of the serotonergic system, was increased in the hypothalamus, PFM and brain stem of animals that received UCN infusions. DOPAC/DA ratio was also increased in the hypothalamus, brain stem and PFM of animals treated with UCN (Figure 22).

Experiment 2: Microdialysis determination of the time course of neurochemical changes in response to icv infusion of UCN. Brain microdialysis can be used to monitor extracellular concentrations of selected compounds after diffusion into the perfusate. The concentrations of compounds recovered in the dialysate are proportional to concentrations immediately outside the dialysis membrane. However, absolute amounts in the extracellular fluid cannot be calculated. Male Sprague-Dawley rats (275-320 g) were stereotaxically implanted with the guide cannulae for the prefrontal cortex microdialysis probe and icv injections. The active length of the dialysis membrane was 4 mm. The perfusion fluid was artificial CSF. Samples were collected for 20 min at a flow rate of 3 ul/min directly into vials containing 30 ul of 0.1M perchloric acid. The samples were frozen soon after collection and stored at -70°C until analyzed. HPLC with electrochemical detection was used to determine the concentration of catecholamines, indolamines and metabolites in the microdialysate. On the day of the experiment, a microdialysis probe was inserted in the guide cannula 2 h before initiating sample collection. Three 20-min samples were collected and used to define the baseline. aCSF was injected icv, and 6 more samples were collected. UCN (3 ug in 3 ul) was then injected and samples were collected for the next 3 hours.

ICV infusion of UCN increased the concentration of DOPAC, 5-HIAA, HVA and MHPG in the microdialysates collected from the medial prefrontal cortex (see Figure 23). DOPAC concentration peaked 2 hours after injection, 5-HIAA and HVA concentrations reached their peak by the third hour after UCN injection. There were no significant differences in NE or MHPG concentrations after UCN infusion suggesting that noradrenergic system to the prefrontal cortex is not activated by UCN infusion. As noradrenergic release in the cortex is supplied exclusively by the locus coeruleus, this implies that the locus coeruleus is not activated by UCN.

Conclusions

These results suggest that UCN, administered centrally, produces neurochemical and neuroendocrine effects similar to those observed after the CRF infusion. However, UCN increased serotonin metabolism whereas other investigators have failed to demonstrate an activation of serotonergic system by icv CRF. The neurochemical pattern of response to UCN

infusion differs from that observed after CRF infusion, as changes are greatly delayed compared to those caused by CRF. With CRF infusions, increased concentrations of metabolites are observed in the first collection sample and peak around 1 hour for most neurotransmitters. The results of the microdialysis experiment show that UCN activates central noradrenergic, serotonergic and dopaminergic systems. Additional experiments are in progress to define neurochemical changes in the medial hypothalamus in response to icv UCN injection. In addition, a cDNA probe for UCN has been developed and Northern blot analysis will be used to determine whether stress changes UCN expression in specific central and peripheral tissues.

Identification of Genetic Markers for Stress Sensitivity and Stress-Induced Behavioral
You Zhou, David Elkins and Igor Rybkin

The objective of this project is to identify potential genetic markers for susceptibility to stress-induced changes in behavior. Five genes that have been reported to be related to either behavior or to activation of the hypothalamic pituitary axis were selected as initial candidate markers. These were angiotensin converting enzyme (ACE), apolipoprotein E (Apo E), corticotrophin releasing hormone (CRH), transforming growth factor α (TGFα) and prolactin (PRL).

Probes for these genes were made by cloning cDNA fragments, obtained by RT-PCR of rat hypothalamic or pituitary tRNA, into plasmid vectors. The identity of the cDNA inserts was confirmed by sequencing and the probes have been tested on Northern blots and appear to detect the corresponding transcripts specifically. Dot blot was used to determine the effect of repeated restraint stress (3 hours/day for 3 days) on expression of the candidate genes in various tissues. Repeated restraint caused changes in expression of TGFa in the hypothalamus, PRL in the pituitary gland and ACE and ApoE in the cerebella cortex.

Although a majority of behavioral studies are carried out using rat models, the availability of molecular and genetic information for rats is still limited compared to that from rapidly developed molecular genetics in mice. In order to take advantage of the transgenic mouse models available behavioral measures for mice had to be developed. Two behavioral tests were established for mice: open field test for locomotion and anxiety-type behavior and a water maze test for spatial memory and learning ability. Two pilot tests have been completed to determine the most sensitive assays for testing behavior in mice.

Experiment 1: Effect Of Repeated Stress On Food Intake, Body Weight, Spatial Memory And Open Field Activity In Mice. A total of 48 adult mice (16 CD1 mice and 32 C57BL/6J mice, 4-6 weeks old, ~18-20 g each) were used for (1) determination of the duration of restraint and for setting up the Morris water maze conditions using white object on dark background (CD1 mice) and using black object on white background (C57BL) ; (2) determination of the effects of repeated restraint stress on feeding behavior, body weight, open field activities, and spatial memory in mice (C57BL). In each experiment, mice were allowed an adaptation to the diet and environment for 6 days before their food intake and body weight were recorded. They were

divided into 2 weight matched groups (8 for restraint and 8 as controls) on day 7 of body weight recording. Food intake and body weight were monitored for 20 days (3 days after the end of the 2nd session of restraint as described below).

Three sessions of restraint were applied between 10-11 a.m. with a 4-day break between sessions. In the 1st session, mice were restrained in a plastic tube for ~20 min on day 8, 9, and 10. They were tested in open field on day 8 and day 9. The 2nd session of repeated restraint began on day 15. The third one was from day 22 to day 25. Mice were also used for Morris water maze test during the 2nd and 3rd session of restraint. Control mice were allowed to stay in their home cage but without food and water for the duration of each restraint period (~20 min).

Results and Discussion

The results showed that the first, but not the second, session of repeated restraint caused a significant inhibition of food intake and loss of body weight in mice. The restraint stress had significant effects on open field activity, with stressed mice showing less anxiety-type activity than controls. Restraint also caused an impairment of reference spatial memory on all days tested and impaired working memory on the first 3 days of repeated restraint.

Experiment 2: Apolipoprotein E: A Potential Candidate As Genetic Marker For Stress Susceptibility. Of the 5 selected candidate genes we first focused on ApoE, one of several lipoprotein transfer genes. ApoE protein is a ligand to the LDL receptor and plays an important role in lipoprotein metabolism by mediating lipoprotein removal from the blood. It has been shown that variation at the ApoE locus is associated with increased risk for developing atherosclerosis. ApoE is associated with development and maintenance of functional integrity of neuronal cells and genetic variation, has recently been linked to Alzheimer's disease. A memory impairment is observed in ApoE-deficit mouse and this deficit can be significantly corrected by central infusion of ApoE, suggesting involvement of ApoE in spatial learning mechanisms. We examined the behavior of both wild-type and ApoE-deficit mice in response to restraint stress.

Materials and Methods

A: 8 control and 8 chronically stressed $ApoE^{+/+}$ or Apo-E deficient mice (C57BL/6J, 4-6 weeks old, ~18-20 g each) were used for open field test and Water Maze task. At the end of this study (9 days after the last restraint experiment), controls were introduced to acute stress and immediately killed. The other 8 mice that were exposed to chronic stress (repeated restraint) before the open field and Maze tests, were killed without acute restraint.

B: 24 $ApoE^{+/+}$ mice were divided into 3 groups: 1) control, 2) chronic stress, and 3) acute stress. Mice were handled daily for 7 days. Group 2 was exposed to a 20-min restraint daily for 3 consecutive days. They were killed a day after the 3rd restraint, along with the controls and the acute stress group of mice that were restrained for 20 min immediately before decapitation.

Results and Discussion

Food Intake and Body Weight. A significant decrease in food intake by $ApoE^{+/+}$ mice exposed to restraint stress was observed on the initial 3 days during the 1st session of restraint. The food intake was back to normal level on day 3 post restraint. No difference in daily food intake was found in ApoE-deficit mice, suggesting that these mice are less responsive, at least in terms of food intake, to restraint stress. In addition, there was no effect of stress on food intake of either genotype during the 2nd session of restraint. The initial restraint stress caused a significant loss of body weight in both genotypes of mice (Fig. 24A). The ApoE-deficit mice, however, recovered the lost weight faster than the wild type mice. Similar results were observed in the 2nd session of restraint (Fig. 24B). This suggests that the delay in gaining back the body weight after the initial session of restraint is not associated directly with their daily food intake during the post stress period.

Open Field Activities. Control mice of both genotypes traveled significantly shorter distance in day 2 than in day 1, but no differences in locomotive activities were observed in mice exposed to restraint stress (Fig. 25A). In the first exposure of mice to a novel environment, restrained mice of both genotypes showed less anxiety-type behavior than controls, traveling further in the center zone of the field (Fig. 25B). There was no effect of stress on distance traveled in the center zone during the second exposure to the same open field. Restraint stress had no effect on rearing behavior away from the dark board of the field, but caused a significant reduction in numbers of leanings against the wall of the field (Fig. 25C). These results indicate that restraint stress has a strong effect on exploratory activity resulting a decreased degree of cautiousness in a risky environment (in this case, the center zone) and a decrease in exploratory activity in a safer environment (the dark corners).

Morris Water Maze Test. Restraint stress resulted in a significant disruption of both reference and working memory of wild-type mice on the first two days ($p<0.05$), but not on the third, suggesting an adaptation to repeated restraint (Fig. 26). There was no effects of restraint on reference memory of ApoE-deficit mice on day 1 and day 2, possibly due to the severe memory impairment in this genotype (Fig. 26A). However, restraint stress did make reference memory worse on day 3. The $ApoE^{-/-}$ mice also showed an impaired spatial working memory when compared to the control $ApoE^{+/+}$ mice and the spatial memory of these transgenic mice was similar to, if not worse than, that of $ApoE^{+/+}$ mice exposed to restraint stress (Fig. 26B). Again, there were no effect of restraint stress on working memory in ApoE-deficit mice.

Taken together with the results from food intake and body weight studies, we believe that ApoE-deficit mice have an altered susceptibility to stress, as they were less responsive to the restraint stress than the control genotype.

Preliminary Results From Northern Analysis Of ApoE Expression. We have started to analyze ApoE mRNA expression in different tissues obtained from the experiments described above. We have focused on the HPA system and related brain areas, especially the

hypothalamus and hippocampus. The preliminary results are shown in Figure 27. Restraint stress appeared to up-regulated ApoE mRNA expression in the hypothalamus and liver of mice exposed to repeated restraint stress. The ApoE mRNA level was higher in the hypothalamus of mice exposed to acute stress before being killed when compared to non-restrained controls, although the differences were not significant. These preliminary results suggest that chronic stress may caused an alteration in ApoE mRNA level. Future studies will investigate the effect of stress on Apo E expression in more detail and will determine whether it can be considered a potential marker for stress responsiveness.

The Effect of Naltrexone on Food Intake and Saccharin Preference In Stressed rats
Ruth Harris and Leigh Howell

We have previously reported that rats exposed to one hour of restraint stress decrease their saccharin intake but do not change their preference for this solution. This suggested that the reduction in saccharin intake may be associated with a reduction in appetite, rather than a direct effect of stress on the sensory perceptions associated with the sweet solution. In order to demonstrate that the reduction in intake was not due to the rewarding properties of the saccharin we treated rats with naltrexone, an opioid receptor antagonist, immediately after they had been exposed to one hour of restraint stress to determine the importance of opioids in determining stress-induced changes in saccharin intake. Naltrexone has previously been shown to suppress consumption of high fat diets and saccharin solutions in non-stressed rats.

Materials and Methods

Twenty eight male Sprague Dawley rats, had free access to powdered chow and water. Daily body weights and food intakes were recorded for 4 days and then the rats were divided into 4 weight matched groups of 7 animals. Two groups of rats acted as controls and two were restrained for 1 hour in plastic restraining tubes from 11.00 a.m. to 12.00 p.m. At the end of the restraint period each animal received an i.p. injection of either saline or 2 mg/Kg Naltrexone dissolved in saline. Thirty minutes later the rats were given free access to food and to two bottles, one containing water and the other containing 100 mM Saccharin solution. Food intake and saccharin preference were measured after 2, 8 and 24 hours. Daily body weights and food intakes were recorded for a further 4 days. Statistically significant differences between groups were determined by two-way analysis of variance or by two-way repeated analysis of variance when comparing daily measurements.

Results

During the 2 hours after stress Naltrexone significantly inhibited water intake and food intake, there was no effect on saccharin intake or saccharin preference and stress had no independent effect on any of the intakes measured. Intakes recorded during the 8 hours following stress are shown in Figure 28. Naltrexone and stress had significant effects on saccharin intake and there was a significant interaction between stress and naltrexone. In rats

treated with saline there was a non-significant reduction in saccharin intake of stressed rats. In naltrexone treated rats the saccharin intake of controls was significantly lower than that of saline treated controls. However, stressed rats treated with naltrexone had the same intake as that of saline treated controls. There was no significant effect of either stress or naltrexone on saccharin preferences. Naltrexone combined with stress caused a significant reduction in food intake. Twenty four hour fluid intakes of the rats were not effected by either stress or naltrexone although there was a progressive reduction in saccharin preference of these rats with time. Daily food intakes and body weights of the rats are shown in Figure 29. Comparison of daily food intakes showed a significant interaction between stress and day as the restrained rats ate less on the day of stress than on either the pre-stress or post-stress days. When comparing body weights of the rats there were significant interactions between naltrexone and day and between stress and day, however, post-hoc comparisons of the groups did not reveal any significant differences.

Conclusions

The results from this experiment confirm our previous conclusion that the reduction in saccharin intake of stressed rats is not associated with stress-induced anhedonia, but may be related to a stress-induced inhibition of appetite. Preferences measured only 2 hours after stress did not show any effect of naltrexone on saccharin preference, however, none of the rats showed any strong preference for the solution during this time as preference ratio was around 50% for all groups. During the 8 hour period after stress, naltrexone treated controls had a significantly reduced saccharin intake compared with other groups and preference ratio remained at 50%, indicating no preference or aversion for the solution. By 24 hours the effect of naltrexone on preference was lost. In contrast, restrained rats treated with naltrexone consumed the same amount of saccharin as saline controls during the 8 hours after stress and showed a progressive decline in saccharin preference over days so that it was significantly reduced post-stress, compared with pre-stress. The food intakes of these rats was significantly reduced on the day of stress, but returned to pre-stress levels the day after stress. These observations suggest a complex interaction between stress-induced mechanisms that suppress food intake and the opioid system. It is possible that the opioid system is activated in response to stress to try and maintain food intake and thus retain homeostasis. Thus blockage of the opioid receptors would result in a greater than normal stress-induced inhibition of food intake. The effect of stress plus naltrexone on saccharin intake is more difficult to explain as saccharin intake increased and preference remained above 70%, suggesting that mechanisms other than opioids were involved in driving sensory responses in these rats.

Due to the unexpected interaction between stress and naltrexone on food intake we will determine whether antagonism of opioid receptors in food deprived rats exaggerates CRF-induced aphagia. If it does it may provide a model in which the mechanisms responsible for suppression of food intake is exaggerated.

D. Manuscripts Published/In Press

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Task 4 - Clinical Studies

A. Overview

In the second quarter of this year we completed our first experiment that evaluated tyrosine, phentermine, amphetamine, caffeine and placebo in a total of 76 subjects. The purpose of the study was to evaluate nutrient and drug effects on attention and cognitive performance during 40 hours of sleep deprivation. We presented preliminary results from that study at the CMNR (see appendix, 18th Quarterly Report).

We began enrollment on our second experiment, "Tyrosine Plus Caffeine vs. Caffeine." After accruing only three patients, this research project was suspended based upon preliminary discussions with the CMNR.

Further activities of this quarter involved data analysis and preparation for publication of the first experiment wherein 76 patients were enrolled.

B. Progress on Completed Projects

The letter report of the CMNR recommended that the project not continue beyond this grant year. Therefore, our efforts in this year focused on completing the first experiment involving 76 subjects and evaluating data from that experiment in preparation for publication.

We initiated a series of manuscript planning meetings. Dr. Richard Magill became ill in November and required neurosurgery and radiotherapy. Thus, he was unable to participate in the sessions until March, 1997. The following manuscript meetings were held: October 1, 1996, October 11, 1996, November 4, 1996, December 3, 1996, December 19, 1996, January 15, 1997, January 22, 1997, January 31, 1997, February 19, 1997, and March 12, 1997. Attendees included Drs. Ryan, Bray, Waters, Magill, Smith, Volaufova and Cindy Hadden, a doctoral candidate in kinesiology.

Three manuscripts were identified for working groups. The first manuscript will be co-authored by Drs. Waters and Magill and will present the analysis of effects on primary endpoints. The second manuscript will be authored by Drs. Bray and Smith and will present the neuroendocrine response to sleep deprivation. The third paper will be authored by Dr. Magill and will evaluate the relative merits of the 20 procedures used to evaluate performance in the experiments.

We finalized the data analysis for the sleep data with primary endpoints being change in multiple sleep latency tests and the presence of three consecutive sleep episodes. We conducted a preliminary analysis of the performance data and the neuroendocrine data. The appendices of the 19th and the 20th Quarterly Reports contain biostatistical summaries.

C. Progress on Ongoing Projects

We closed the sleep laboratory in the PBRC residence hall. We will not continue nutritional neuroscience clinical research studies based upon the recommendation of the CMNR.

D. Manuscripts Published/In Press

None.

E. Manuscripts in Preparation

See above.

F. Abstracts

None.

V. Menu Modification Project

A. Overview

The current Menu Modification Project is the continued development of recipes which enable the design of Army garrison menus which meet specified nutrition targets of lower fat, cholesterol, and sodium. This has been an initiative of the Armed Forces Recipe Service, the Army Master Menu and the Army Food Service Program since 1985. Specifically the project involves developing recipes which are lower in fat to achieve a fat target of 30% of total energy from fat, providing soldiers low cholesterol alternatives to eggs aimed at a total daily intake of cholesterol of approximately 300 mg/day, and reducing sodium in recipes. From our experiences with the initial acceptability and consumption studies and positive feedback from USARIEM, we proposed to continue to develop recipes and plan to further proceed with the Menu Modification Project. The project includes:

1. Development of 10-15 new recipes per quarter.

   LTC Cline and K. Patrick plan to visit the Quartermaster School in Fort Lee, VA in Summer, 1997 to discuss recipe development and a potential module for low-fat cooking techniques to incorporate into the Cook's School training program.

2. Verification of new recipes at Louisiana Tech under the direction of Dr. Alice Hunt.

3. Acceptability study of new recipes at Fort Polk or other designated Army post. Fort Lee might provide an ideal situation since the Quartermaster School is located there.

B. Progress on Completed Projects

Data from the acceptability study was presented at the Experimental Biology >96 meeting on April 15, 1996, in Washington, DC. These data were also presented at the 21st National Nutrient Databank Conference held in Baton Rouge, LA June 20-22, 1996. Information on the posters can be found in the Quarterly Report of 7/96.

Seventeen new recipes developed and tested by Kelly Patrick were sent to Dr. Alice Hunt at Louisiana Tech University to be validated. The recipes were prepared to yield 100 portions and offered to students and staff for evaluation. A slightly modified version of the food evaluation form was used to determine acceptability of the menu items. The computerized optical mark sense food evaluation forms previously used by Louisiana Tech were discontinued and data was manually entered into an Excel spreadsheet. The acceptability scores were determined and a report was included in the Quarterly Report 10/96. Overall, the scores exceeded a rating of 5.0, with 15 recipes scoring above 5.9. Louisiana Tech continues to be an

asset in this project in the evaluation of the directions, format, and quantity of each recipe during preparation.

In our annual report of April 1996, a visit to Natick to meet with the USARIEM contingent there and acceptability experts was in the planning. This visit did occur and involved Kelly Patrick, Baldwin Sanders, Ray Alen, Pat Marquette, and Catherine Champagne. The visit was coordinated by LTC Cline and details concerning the issues discussed can be found in the Quarterly Report 12/96. Overall, the visit was productive and will assist in better planning of acceptability testing procedures.

PBRC served as nutrition support for the Field Study with volunteers from the 1/75th Ranger Regiment, Hunter Army Airfield, GA. The study took place from 13 July - 5 August, 1996. A number of personnel, including staff and students were able to support the study which involved garrison dietary intakes, food records, and MRE collections.

We also assisted as nutrition support for the Field Study at the three phase study at the Sergeants Major Academy, Biggs Army Airfield, Fort Bliss, El Paso, TX from 19 - 26 September, 1996, 7-13 December, 1996 and 1-7 March, 1997. For this study, we were assisted by dietetic interns from two internship programs in Louisiana (North Oaks and Louisiana Tech) and personnel from other departments within the PBRC.

C. Progress on Ongoing Projects

The recently renovated kitchen in the Conference Center will provide a more efficient site for future recipe development. Two rooms adjacent to the kitchen and Kelly Patrick' s office will be used for the testing procedures. This kitchen will also provide a good environment for visual estimation and recipe specialist training and was utilized for these trainings for the Savannah study.

Kelly Patrick continues to develop recipes. Kelly Patrick suggested several ideas for new recipes and presented these to the CMNR Site visit September 18, 1996. We plan to continue the validation of the recipes by Louisiana Tech. The recipes are described in Quarterly Report 10/96.

The issues of software development, licensing, and other related matters discussed at the October visit to Natick are currently being addressed. We are coordinating efforts with USARIEM-Military Nutrition Division dietitians and data support personnel. In conjunction with these matters, Ray Allen, Pat Marquette, and Catherine Champagne are involved. Future support includes a study of. Assessment of Nutritional Status and Energy Expenditures and Determination of Gender Differences in Dietary Intakes of Combat Service Support Personnel Subsisting on Meal-Focused Versions of the Meal, Ready to Eat@ to be conducted at Fort Bragg/Camp McCall from 30 April to 13 May, 1997.

D. Manuscripts Published/In Press

None to report at this time.

E. Manuscripts in Preparation

Champagne CM, A Hunt, AD Cline, K Patrick, and DH Ryan. Acceptability of new recipes for modification of military menus.

F. Abstracts

Acceptability of new recipes for modification of military menus. C Champagne, A Hunt, A Cline, and K Patrick (Spon: D York). Pennington Biomedical Research Center, Baton Rouge, LA 70808, Louisiana Tech University, Ruston, LA 71272 and USARIEM-MND, Natick, MA 01760. Experimental Biology >96, Washington, DC, April 15, 1996.

Comparison of acceptability scores of modified recipes among test settings. A Hunt, A Cline, C Champagne, K Patrick, and D Ryan. Louisiana Tech University, Ruston, LA 71272, USARIEM-MND, Natick, MA 01760, and Pennington Biomedical Research Center, Baton Rouge, LA 70808.

VI. Metabolic Unit Project

A. Overview

Two projects will be discussed. One experiment was executed in 1993.

B. Progress on Ongoing Projects

During the second year of this grant, activity in this project consisted of carrying out an experiment on two cohorts of Special Operation Forces volunteers. That project was, **"Assessment of Intra- and Inter- Individual Metabolic Variation in Special Operations Forces Soldiers."** The Principal Investigator for the project is Ms. T. E. Jones affiliated with the Military Nutrition Division at USARIEM. Co-Investigators are C. Gabaree, Lt. Col. T. C. Murphy, Donna Ryan, M.D., E. Brooks, R.N., M.N.

The purpose of the study was to evaluate a group of Special Operations Forces volunteers to determine the metabolic variation during rest, exercise and post-exercise recovery of the individual soldiers. The complete amended protocol can be found in Appendix VI of the Fifth Quarterly Report. On June 11 ten SOF soldiers arrived to serve as the first cohort for testing. Army personnel at the PBRC included Tanya Jones, Principal Investigator, Sven Ljamo, M.D. (medical monitor), Catherine Gabaree (exercise physiologist), Lt. Col. Cliff Murphy (dietician) and three civilian spotters for exercise testing. The first cohort of military volunteers and civilians left the PBRC on July 1, 1993. There were minimal complications that occurred in the SOF volunteers (subungual hematomas, muscle soreness, poison ivy dermatitis). All procedures were carried out safely and satisfactorily. A mid-course correction session at the end of the first cohort stay resulted in minor procedure adjustments. From July 9-24, 1993 ten members of the Special Operations Forces from the 10th SFG at Fort Devens,

Massachusetts participated in the study. All procedures were carried out safely and satisfactorily.

In the last quarter of the third year of the project we discussed the publication process with Dr. Harris Lieberman. PBRC scientists agreed to assist USARIEM in the completion of the draft manuscript begun by Ms. Tanya Jones.

Also in the 12th quarter, planning began on a new metabolic unit project, "Effects of Prolonged Inactivity on Musculoskeletal and Cardiovascular Systems with Evaluation of a Potential Countermeasure." At the January 26, 1995 site visit, Drs. Vogel and Lieberman and Col. Gifford agreed to support the development of a protocol to evaluate the use of a pharmacologic countermeasure to the physical and functional effects of prolonged inactivity. However, based upon review by the CMNR (see appendix of 19th and 20th Quarterly Reports), we decided not to proceed with this project.

C. Progress on Ongoing Projects

None.

D. Manuscripts Published/In Press

None

E. Manuscripts in Preparation

"Assessment of Intra- and Inter-Individual Metabolic Variation in Special Operations Forces Soldiers."

F. Abstracts

None.

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APPENDIX A

CLINICAL LABORATORY FOR HUMAN AND FOOD SAMPLES

Method C:\HPCHEM\1\METHODS\JRAA.M

linearity

Calibration Table

amino acids

Calib. Data Modified : Wednesday, March 26, 1997 8:43:20 AM

Calculate : Internal Standard
Based on : Peak Area

Rel. Reference Window : 2.000 %
Abs. Reference Window : 0.000 min
Rel. Non-ref. Window : 2.000 %
Abs. Non-ref. Window : 0.000 min
Uncalibrated Peaks : not reported
Partial Calibration : Yes, identified peaks are recalibrated
Correct All Ret. Times: No, only for identified peaks

Curve Type : Linear
Origin : Included
Weight : Equal

Recalibration Settings:
Average Response : Average all calibrations
Average Retention Time: Floating Average New 75

Calibration Report Options :
Printout of recalibrations within a sequence:
Calibration Table after Recalibration
Normal Report after Recalibration
If the sequence is done with bracketing:
Results of first cycle (ending previous bracket)

Default Sample ISTD Information (if not set in sample table):

ISTD #	ISTD Amount [ng/ul]	Name
1	100.00000	norvaline

Signal 1: FLD1 A, Ex=230, Em=389

RetTime [min]	Sig	Lvl	Amount [ng/ul]	Area	Amt/Area	Ref	Grp	Name
6.023	1	1	10.00000	1.12091	8.92136	1		threonine
		2	50.00000	6.25518	7.99338			
		3	100.00000	12.46216	8.02429			
		4	250.00000	31.47188	7.94360			
		5	500.00000	63.43518	7.88206			
		6	1000.00000	116.07822	8.61488			
6.189	1	1	10.00000	1.38547	7.21775	1		histidine
		2	50.00000	6.62704	7.54485			
		3	100.00000	13.29952	7.51907			
		4	250.00000	33.44690	7.47453			
		5	500.00000	68.25599	7.32536			
		6	1000.00000	125.68697	7.95627			
8.332	1	1	10.00000	1.74450	5.73230	1		tyrosine
		2	50.00000	9.18721	5.44235			

Method C:\HPCHEM\1\METHODS\JRAA.M

RetTime [min]	Sig	Lvl	Amount [ng/ul]	Area	Amt/Area	Ref	Grp	Name
		3	100.00000	18.55900	5.38822			
		4	250.00000	46.72025	5.35100			
		5	500.00000	95.63004	5.22848			
		6	1000.00000	176.53600	5.66457			
9.964	1	1	10.00000	1.90959	5.23672	1		valine
		3	100.00000	19.02084	5.25739			
		4	250.00000	46.41557	5.38612			
		5	500.00000	92.44022	5.40890			
		6	1000.00000	170.74000	5.85686			
10.097	1	1	10.00000	2.19443	4.55700	1		methionine
		2	50.00000	11.65809	4.28887			
		3	100.00000	20.71087	4.82838			
		4	250.00000	52.10151	4.79833			
		5	500.00000	104.00468	4.80748			
		6	1000.00000	190.75189	5.24241			
10.350	1	1	100.00000	18.53997	5.39375	I1		norvaline
		2	100.00000	17.67103	5.65898			
		3	100.00000	19.02192	5.25709			
		4	100.00000	18.72827	5.33952			
		5	100.00000	18.80916	5.31656			
		6	100.00000	17.65994	5.66253			
10.482	1	1	10.00000	4.01468	2.49086	1		tryptophan
		2	50.00000	15.34471	3.25845			
		3	100.00000	29.64264	3.37352			
		4	250.00000	73.36302	3.40771			
		5	500.00000	147.15887	3.39769			
		6	1000.00000	266.40015	3.75375			
10.689	1	1	10.00000	2.31584	4.31809	1		phenylalanine
		2	50.00000	11.35741	4.40241			
		3	100.00000	23.46380	4.26188			
		4	250.00000	57.81483	4.32415			
		5	500.00000	115.23796	4.33885			
		6	1000.00000	209.88824	4.76444			
10.905	1	1	10.00000	1.91326	5.22667	1		isoleucine
		2	50.00000	23.61290	2.11749			
		3	100.00000	36.52074	2.73817			
		4	250.00000	69.52090	3.59604			
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		6	1000.00000	213.30339	4.68816			
11.222	1	1	10.00000	2.23763	4.46901	1		leucine
		2	50.00000	10.93562	4.57221			
		3	100.00000	21.50640	4.64978			
		4	250.00000	53.34089	4.68684			
		5	500.00000	107.79233	4.63855			
		6	1000.00000	195.17587	5.12358			

3 Warnings or Errors :

Warning : Overlapping peak time windows at 9.964 min, signal 1
Warning : Overlapping peak time windows at 10.35 min, signal 1
Warning : Overlapping peak time windows at 10.482 min, signal 1

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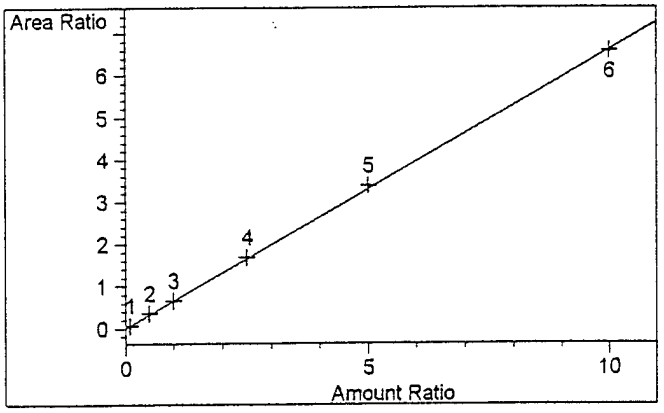
Calibration Curves



threonine at exp. RT: 6.023
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Correlation: 0.99991
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Formula: y = mx + b
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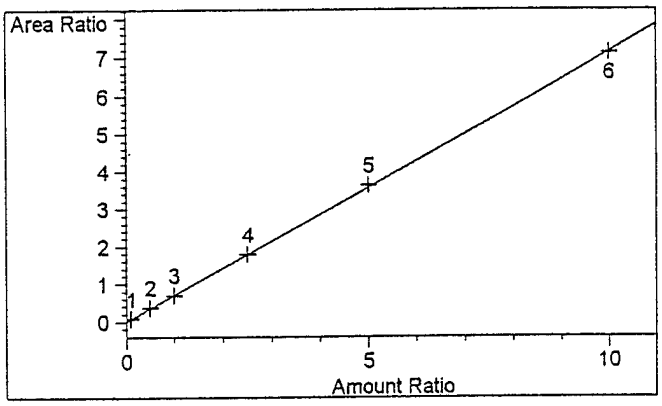
b: 1.51434e-2
x: Amount Ratio
y: Area Ratio



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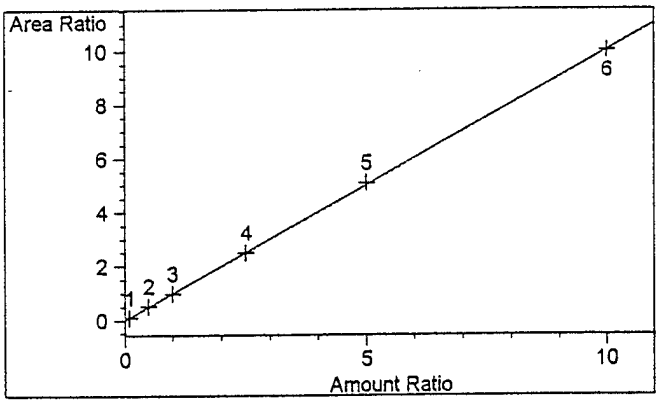
y: Area Ratio



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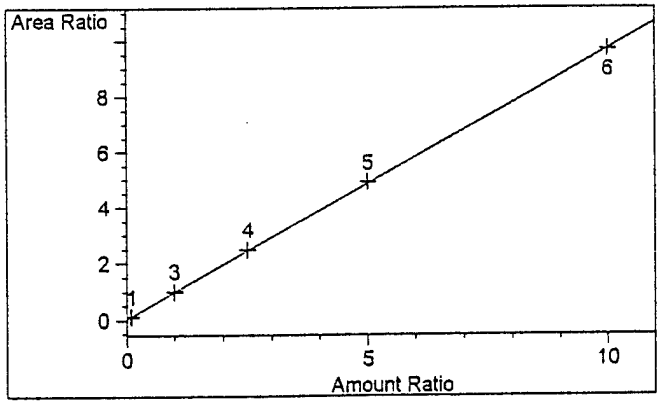
y: Area Ratio



valine at exp. RT: 9.964
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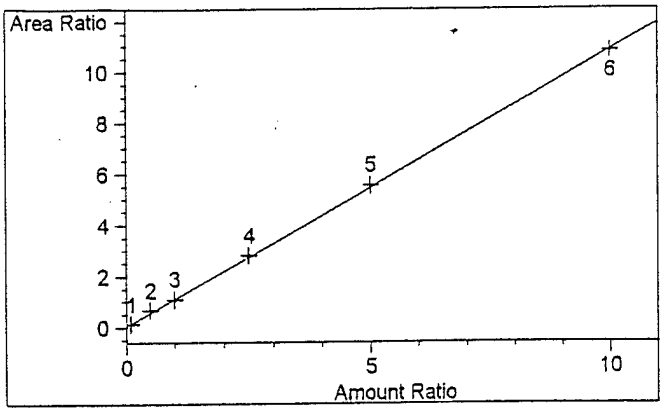
y: Area Ratio



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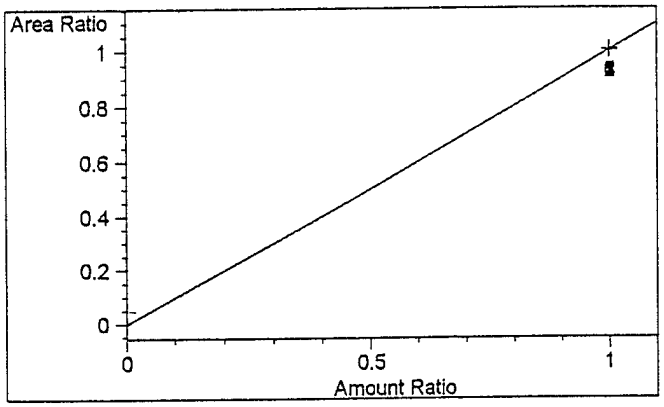
x: Amount Ratio
y: Area Ratio



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Residual Std. Dev.: 0.00000
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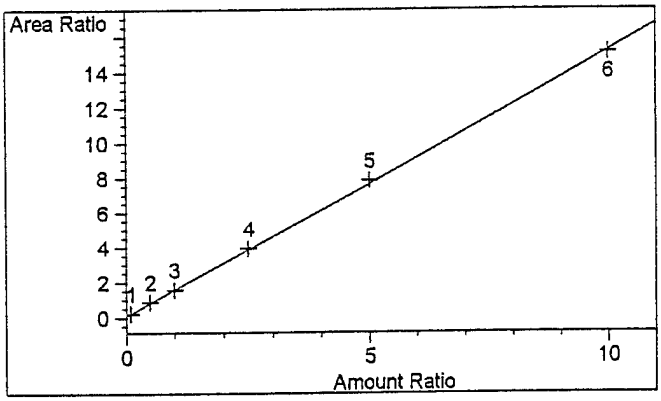
x: Amount Ratio
y: Area Ratio



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Correlation: 0.99984
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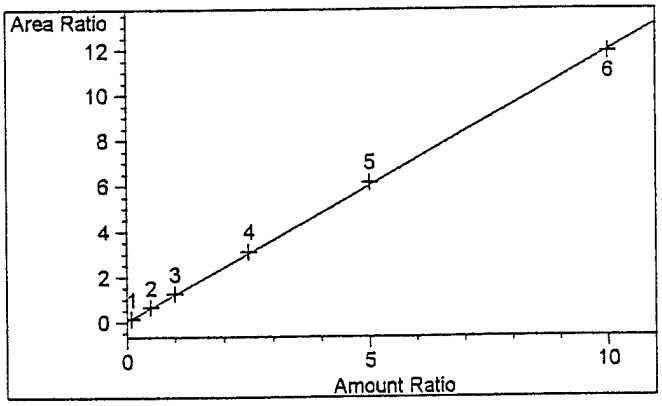
y: Area Ratio



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y: Area Ratio

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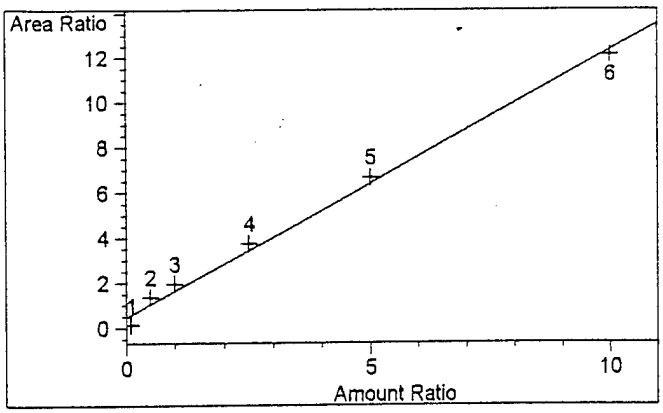

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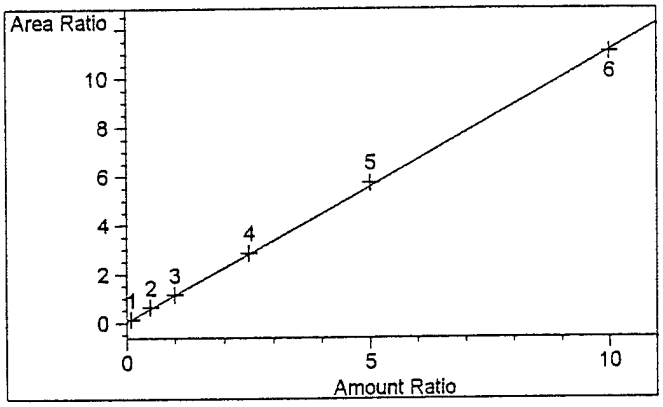
y: Area Ratio



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x: Amount Ratio
y: Area Ratio

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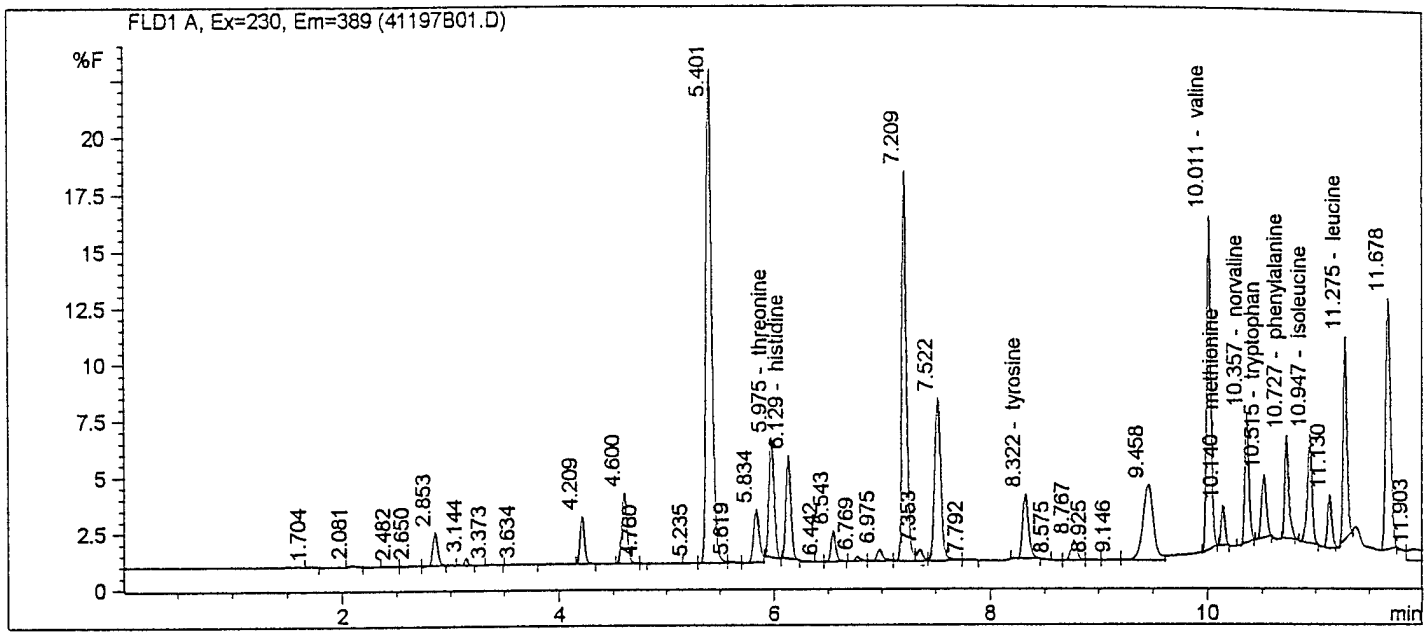
jennifer
Injection Date: 4/11/97 11:28:53 AM
vial: 1
Sample ID: con

sample chromatogram



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1		0.000	1.704	0.038	0.000
2		0.000	2.081	0.258	0.000
3		0.000	2.482	0.052	0.000
4		0.000	2.650	0.100	0.000
5		0.000	2.853	5.031	0.000
6		0.000	3.144	0.845	0.000
7		0.000	3.373	0.108	0.000
8		0.000	3.634	0.199	0.000
9		0.000	4.209	6.138	0.000
10		0.000	4.600	11.338	0.000
11		0.000	4.760	0.043	0.000
12		0.000	5.235	0.105	0.000
13		0.000	5.401	81.807	0.000
14		0.000	5.619	0.386	0.000
15		0.000	5.834	9.006	0.000
16	threonine	178.082	5.975	17.900	1.498
17	histidine	135.290	6.129	14.651	1.390
18		0.000	6.442	0.121	0.000
19		0.000	6.543	4.448	0.000
20		0.000	6.769	0.959	0.000
21		0.000	6.975	2.284	0.000
22		0.000	7.209	52.099	0.000
23		0.000	7.353	1.892	0.000
24		0.000	7.522	29.106	0.000
25		0.000	7.792	0.264	0.000
26	tyrosine	73.501	8.322	11.138	0.993
27		0.000	8.575	0.127	0.000
28		0.000	8.767	3.754	0.000
29		0.000	8.925	0.344	0.000
30		0.000	9.146	0.475	0.000
31		0.000	9.458	23.443	0.000

Data File name: C:\HPCHEM\1\DATA\41197B01.D
Method name: C:\HPCHEM\1\METHODS\JRAA.M

#	Compound	ug/dl	RT	Area	Resp Fact
32	valine	272.999	10.011	40.189	1.023
33	methionine	21.962	10.140	4.339	0.762
34	norvaline	100.000	10.357	15.055	1.000
35	tryptophan	32.781	10.515	8.816	0.560
36	phenylalanine	64.692	10.727	12.360	0.788
37	isoleucine	44.656	10.947	14.652	0.459
38		0.000	11.130	6.493	0.000
39	leucine	130.342	11.275	22.469	0.873
40		0.000	11.678	36.222	0.000
41		0.000	11.903	4.232	0.000

threonine	178.1
histidine	135.3
tyrosine	73.5
valine	273.0
methionine	22.0
norvaline	100.0
tryptophan	32.8
phenylalanine	64.7
isoleucine	44.7
leucine	130.3

Amino Acid Method Evaluation

3/26/97

Accuracy

50 ng/uL

	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
Actual	50	50	50	50	50	50	50	50	50
observed	49.9	50.7	50.4	50.9	49.4	47.8	49.8	55.9	51.6
%	99.8	101.4	100.8	101.8	98.8	95.6	99.6	111.8	103.2

100 ng/uL

	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
Actual	100	100	100	100	100	100	100	100	100
observed	95.6	96.4	100.4	110.4	100.9	99.4	98.5	95.4	98.3
%	95.6	96.4	100.4	110.4	100.9	99.4	98.5	95.4	98.3

250 ng/uL

	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
Actual	250	250	250	250	250	250	250	250	250
observed	251.9	252.4	253	249.8	245.2	254.9	253.9	245.6	253.8
%	100.76	100.96	101.2	99.92	98.08	101.96	101.56	98.24	101.5

500 ng/uL

	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
Actual	500	500	500	500	500	500	500	500	500
observed	512.2	511.4	516.8	520.9	515.2	520.9	516.7	504.9	517.6
%	102.44	102.28	103.36	104.18	103	104.18	103.34	100.98	103.5

1000 ng/uL

	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
Actual	1000	1000	1000	1000	1000	1000	1000	1000	1000
observed	984.4	997.3	1015.2	985.2	988.9	989.9	999.3	961.2	992
%	98.44	99.73	101.52	98.52	98.89	98.99	99.93	96.12	99.2

Precision

Run #	Thr	His	Tyr	Val	Meth	Tryp	Phe	Ile	Leu
1	97	98.1	101.7	101.2	103.4	100.1	102.5	116.5	98.7
2	96.5	97.1	101.2	95.3	98.5	98.8	101.6	113	97.8
3	96.7	97.2	101.7	98.5	100.5	99.8	101.6	112.8	98.1
4	95.9	96.5	101	96	98.4	99.1	101.3	110	97.2
5	94.7	96.3	101.2	97.7	98.9	98.8	101.3	110	97.4
6	96.2	97	101.6	100.2	101.7	99.5	101.2	111.4	98.2
7	95.4	96.6	101.6	99.4	100.8	99.3	102.1	111.3	97.1
mean	96.1	97.0	101.4	98.3	100.3	99.3	101.7	112.1	97.8
s.d	0.8	0.6	0.3	2.2	1.9	0.5	0.5	2.3	0.6
cv	0.8	0.6	0.3	2.2	1.9	0.5	0.5	2.0	0.6

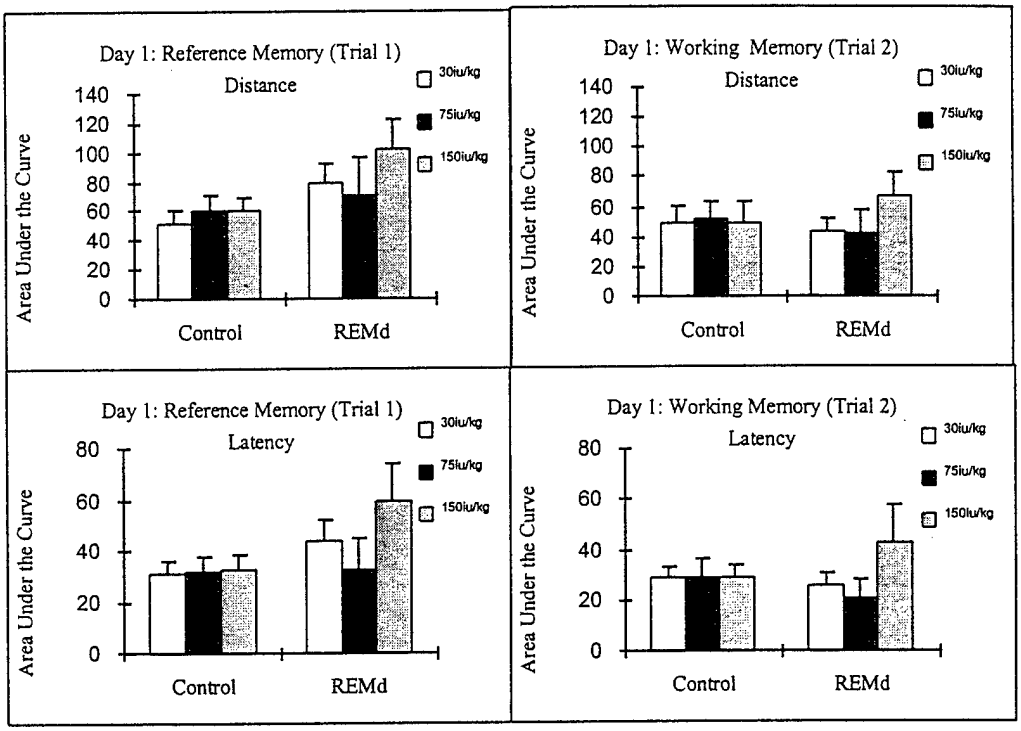
APPENDIX B

NUTRITIONAL NEUROSCIENCES LABORATORY

Figures 1 - 16; 19 - 29

Figure 1: Water Maze Performance of Rats Fed Diets of Different Vitamin E Concentration and Subjected to 48 Hours of Sleep Deprivation

The Effect of 24 Hours REMd on Spatial Memory



The Effect of 48 Hours REMd on Spatial Memory

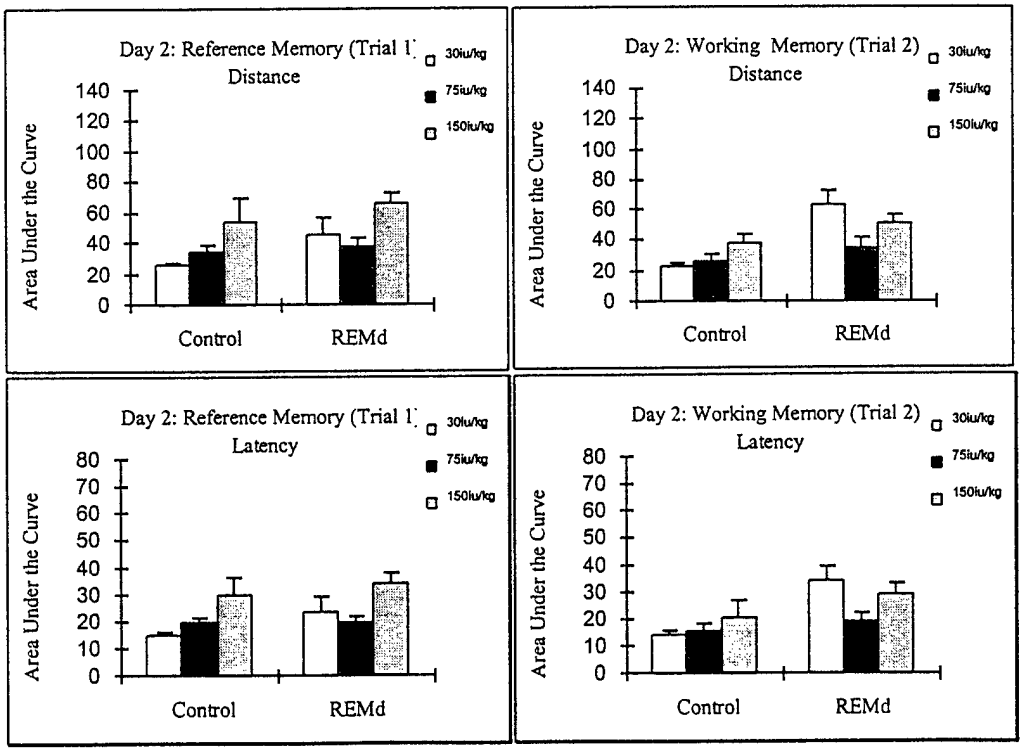
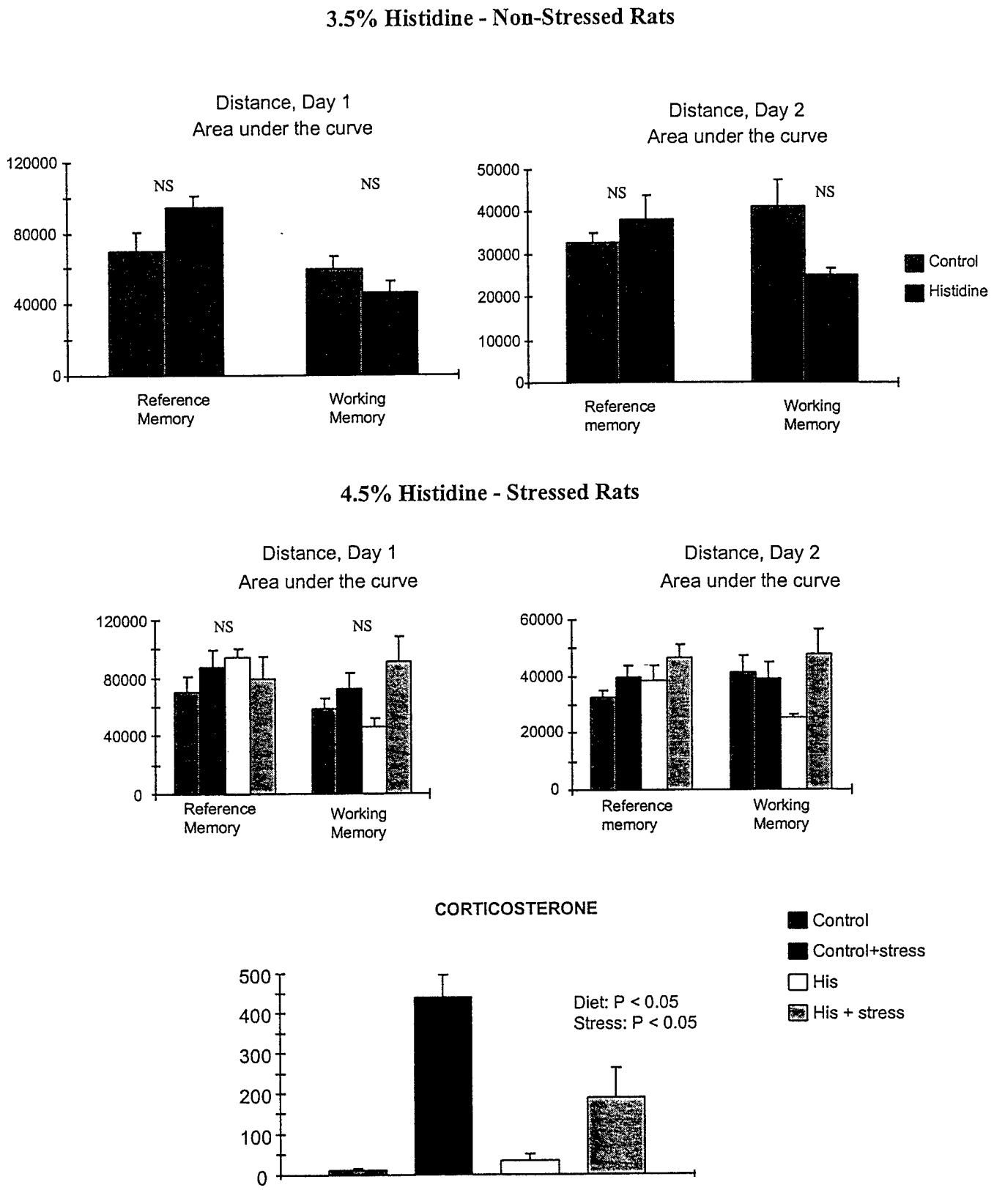
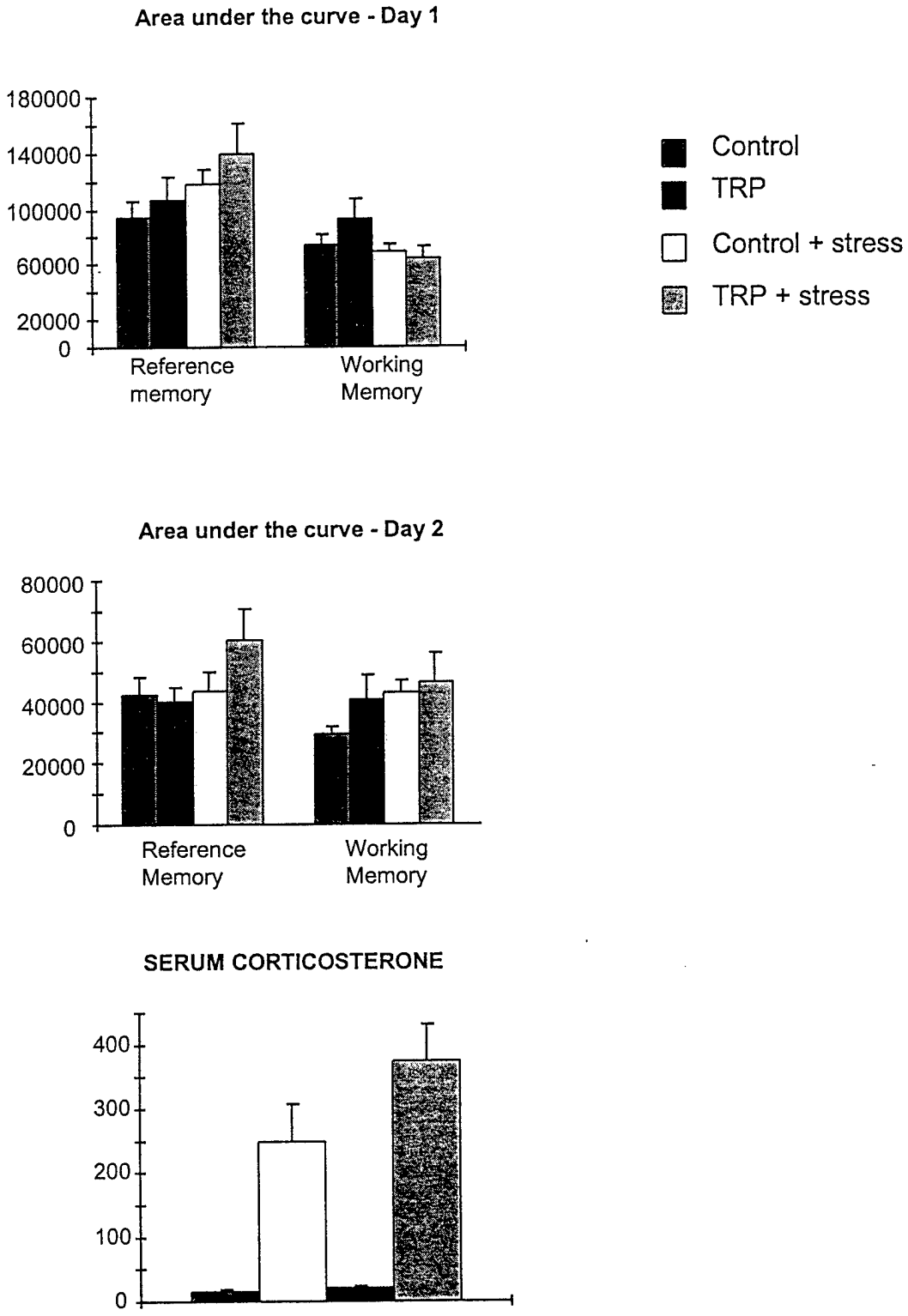


Figure 2: Morris Water Maze Performance of Rats Fed a Histidine Supplemented Diet



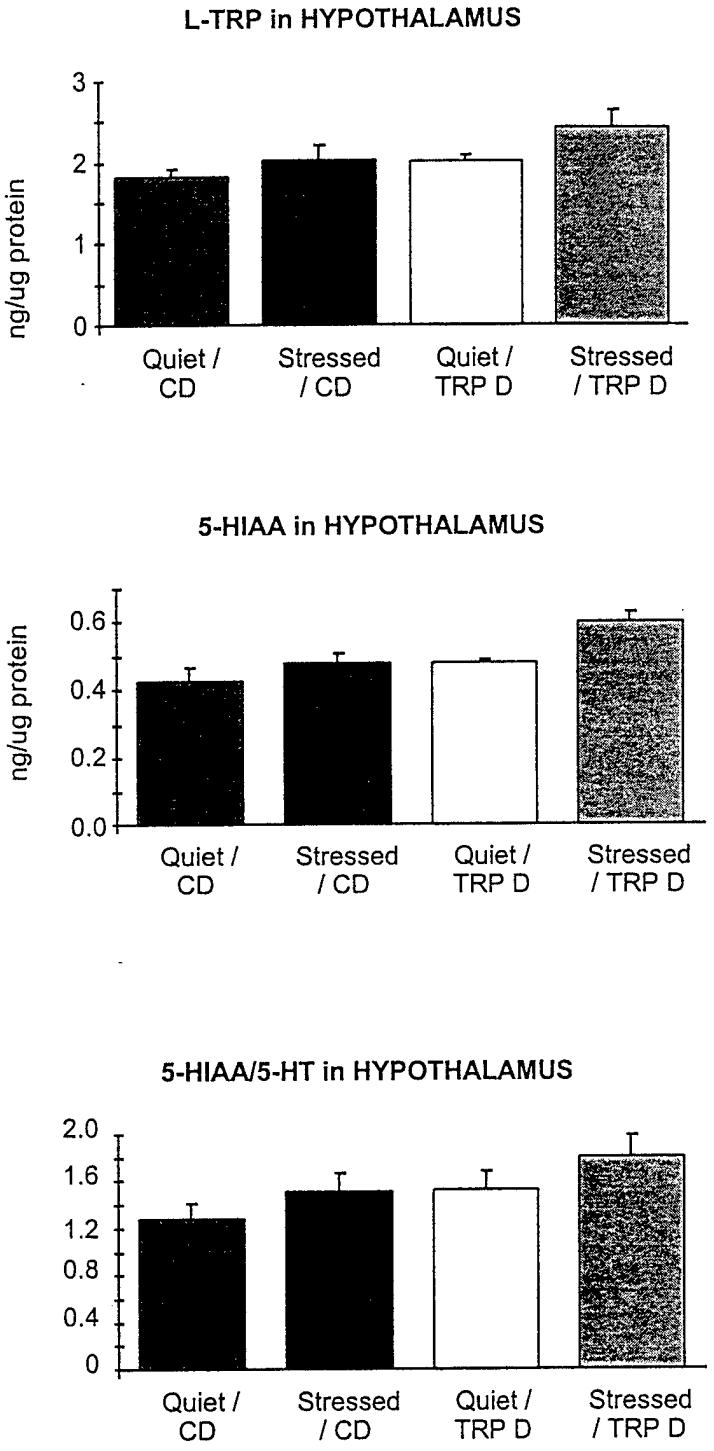
Data are means ± sem for groups of 6 rats. Significant differences were determined by two-way ANOVA

Figure 3: Morris Water Maze Performance of Rats Fed a Tryptophan Supplemented Diet



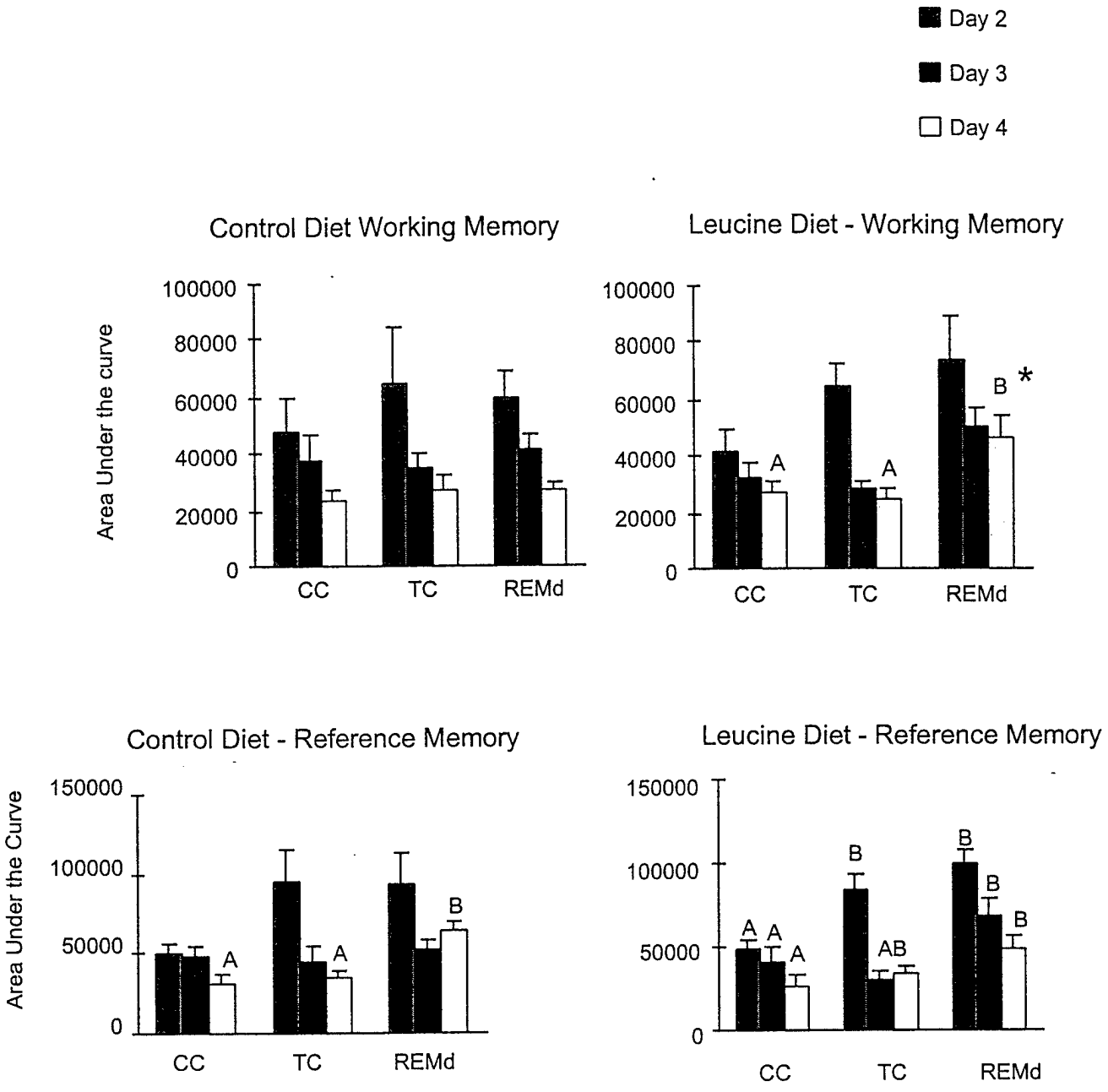
Data are means + sem for groups of 6 rats. There was nor effect of diet or stress on working or reference memory, measured in the Morris Water Maze. There was no effect of diet but a significant effect of stress on serum corticosterone.

Figure 4: Hypothalamic Tryptophan and Serotonin in Rats fed a Tryptophan Supplemented Diet



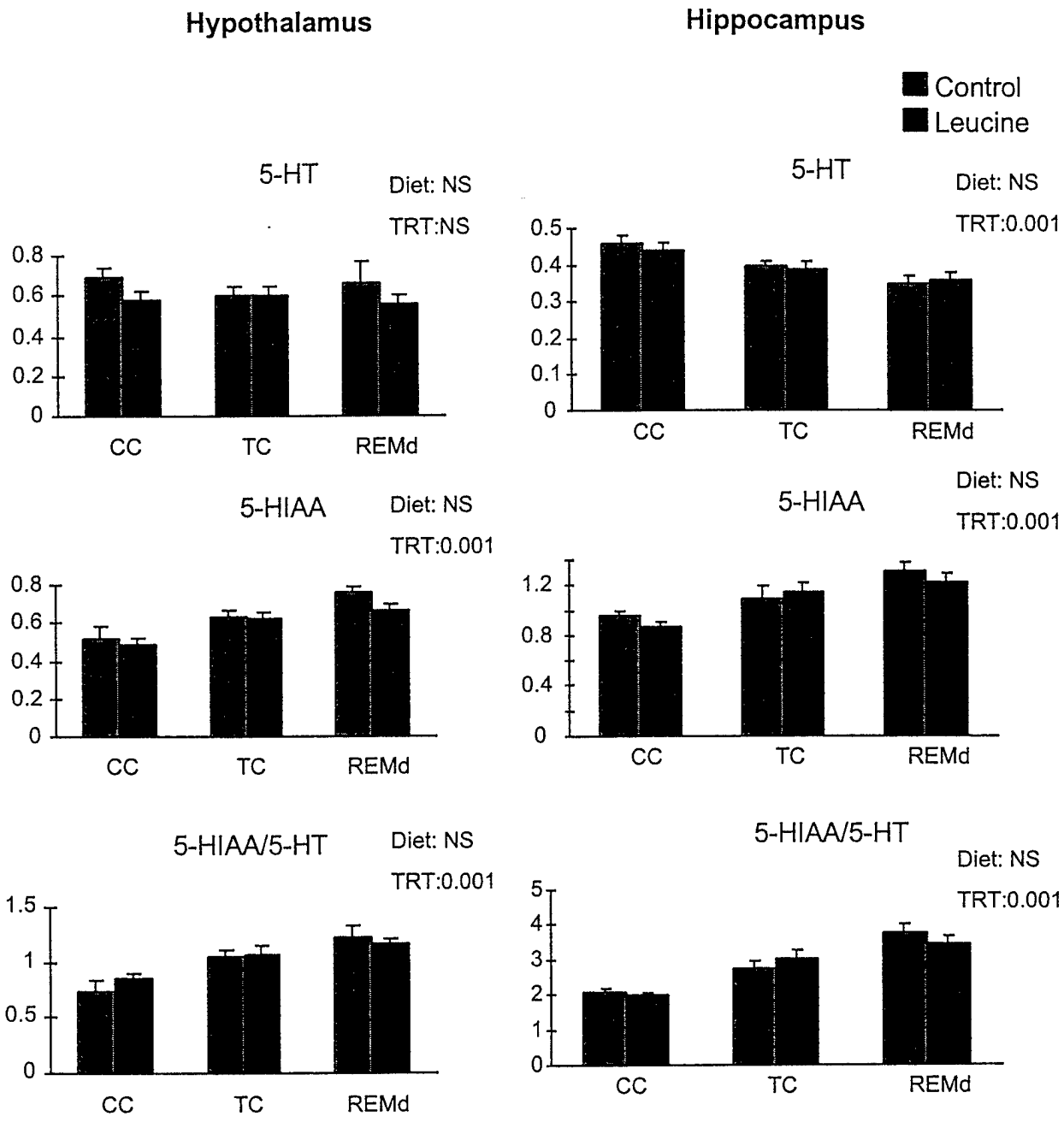
Data are means ± sem for groups of 6 rats. There was a significant effect of diet on Tryptophan and 5-HIAA concentrations. Restraint caused a significant increase in serotonin metabolism (5-HIAA?5-HT).

Figure 5: Water Maze Performance of Sleep Deprived Rats Fed Control or 3% Leucine Diets



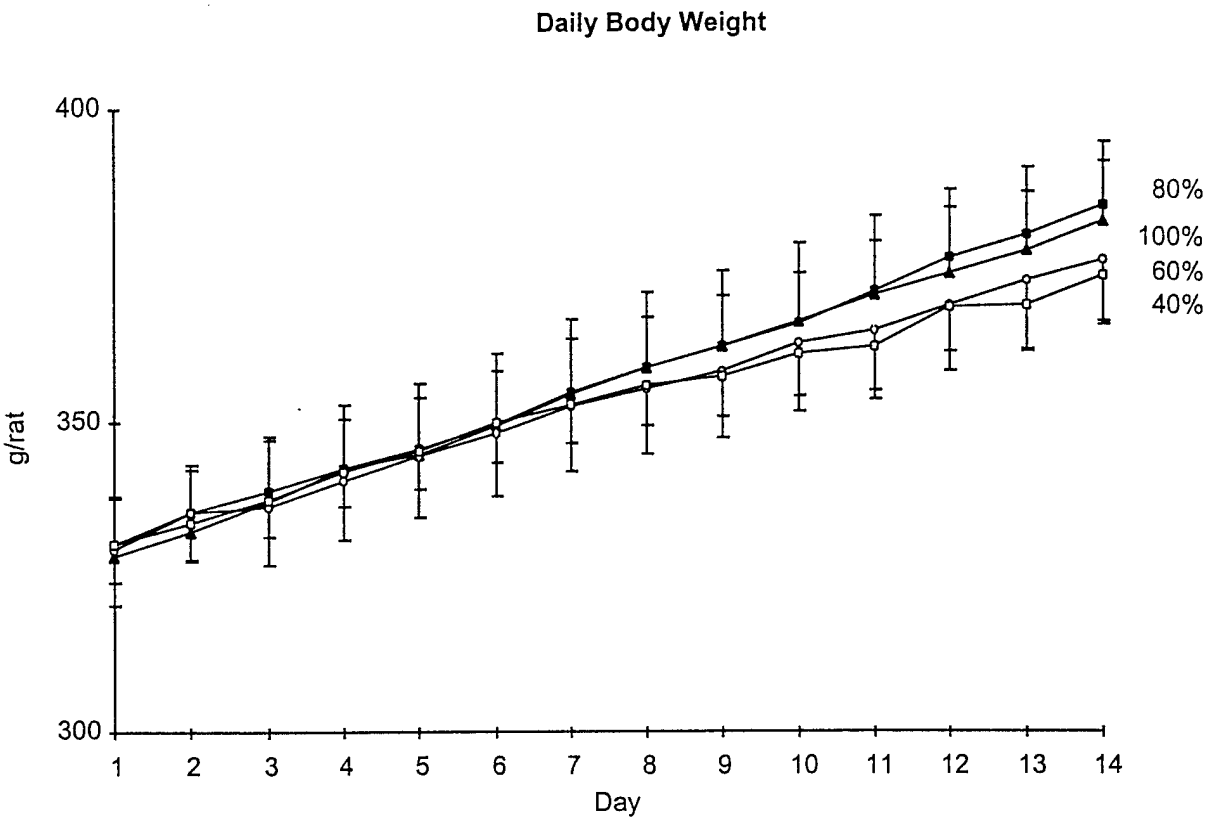
Data are means + sem for groups of 8 rats. Statistical differences were determined by two-way ANOVA with post-hoc t-test. An asterisk indicates a significant effect of diet. Superscripts indicate a significant effects of treatment.

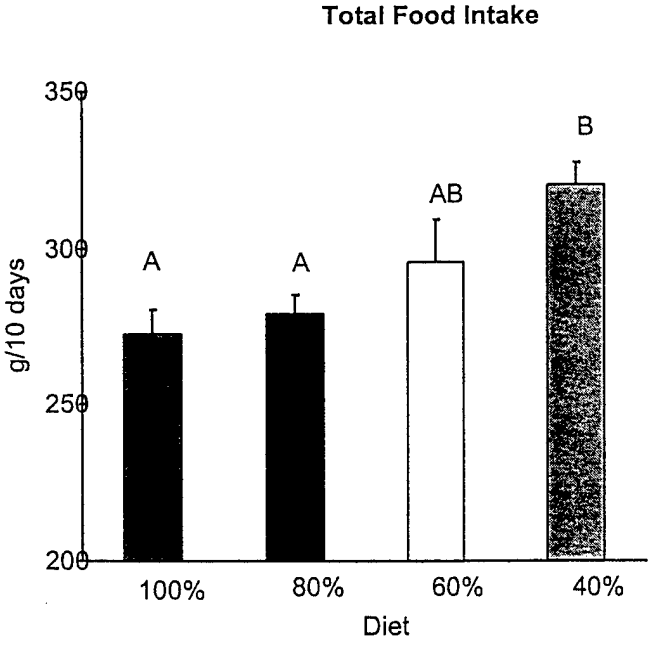
Figure 6: Hypothalamic and Hippocampal Serotonin Metabolism in Sleep Deprived Rats Fed a Diet Containing 3% Leucine

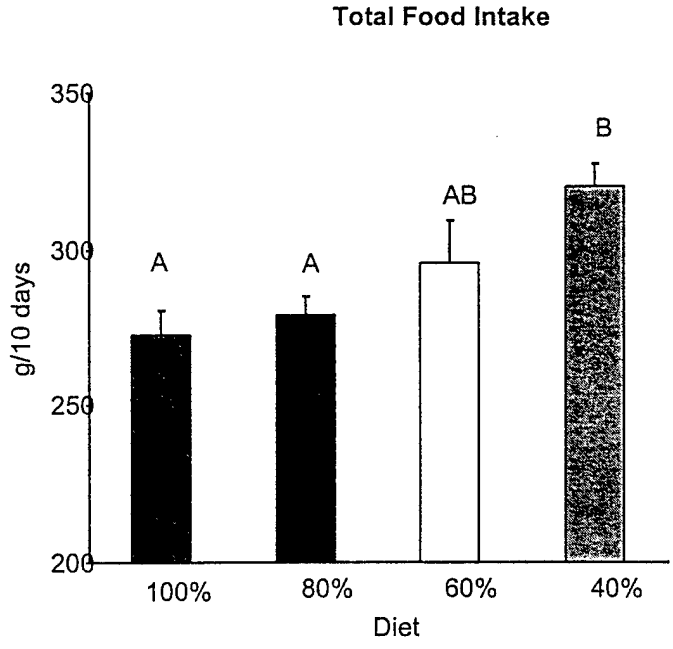


Data are means + sem for groups of 8 rats. Statistical differences were determined by two-way ANOVA

Figure 7: Daily food intakes and body weights of rats fed diets of different amino acid content

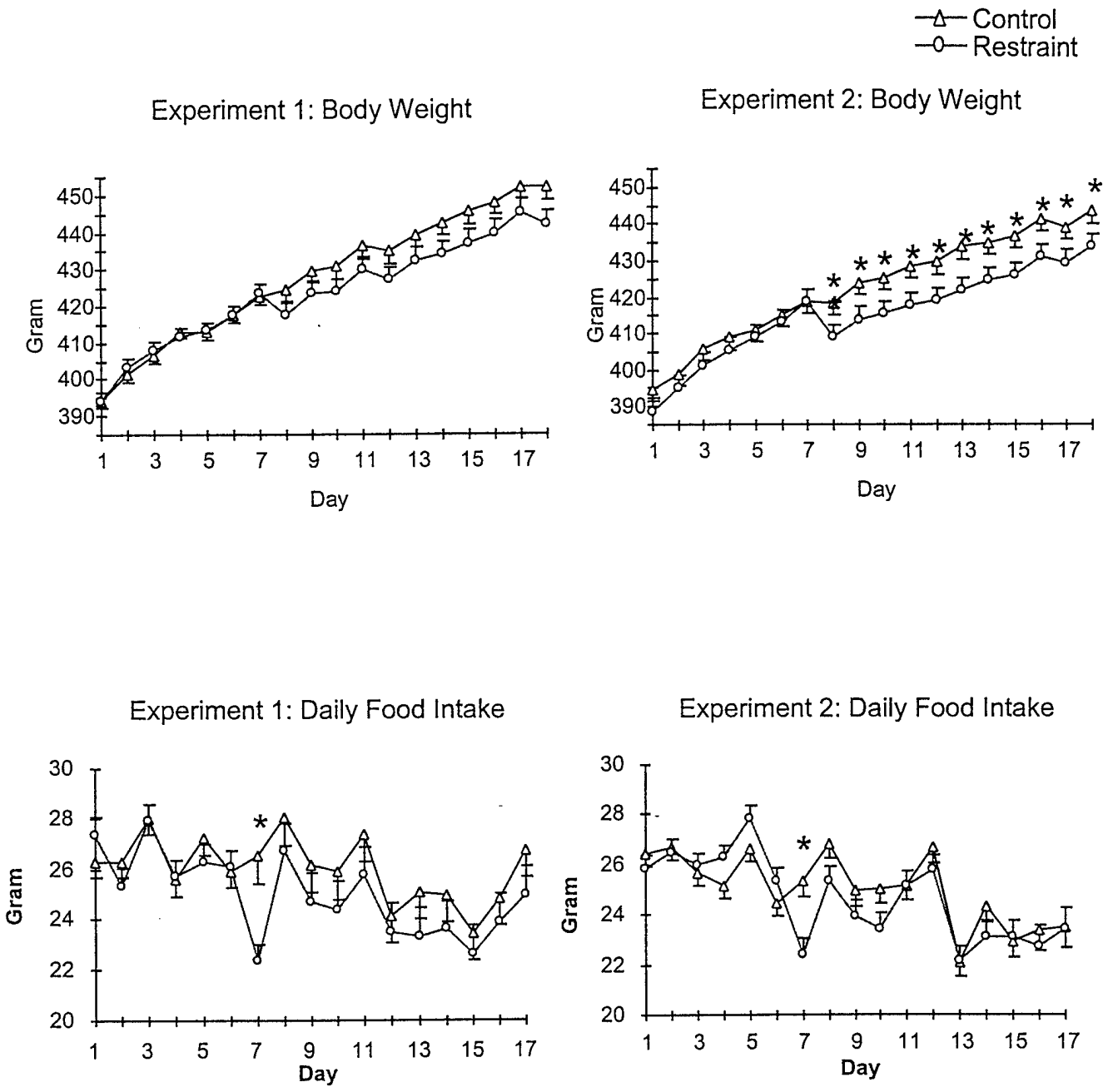






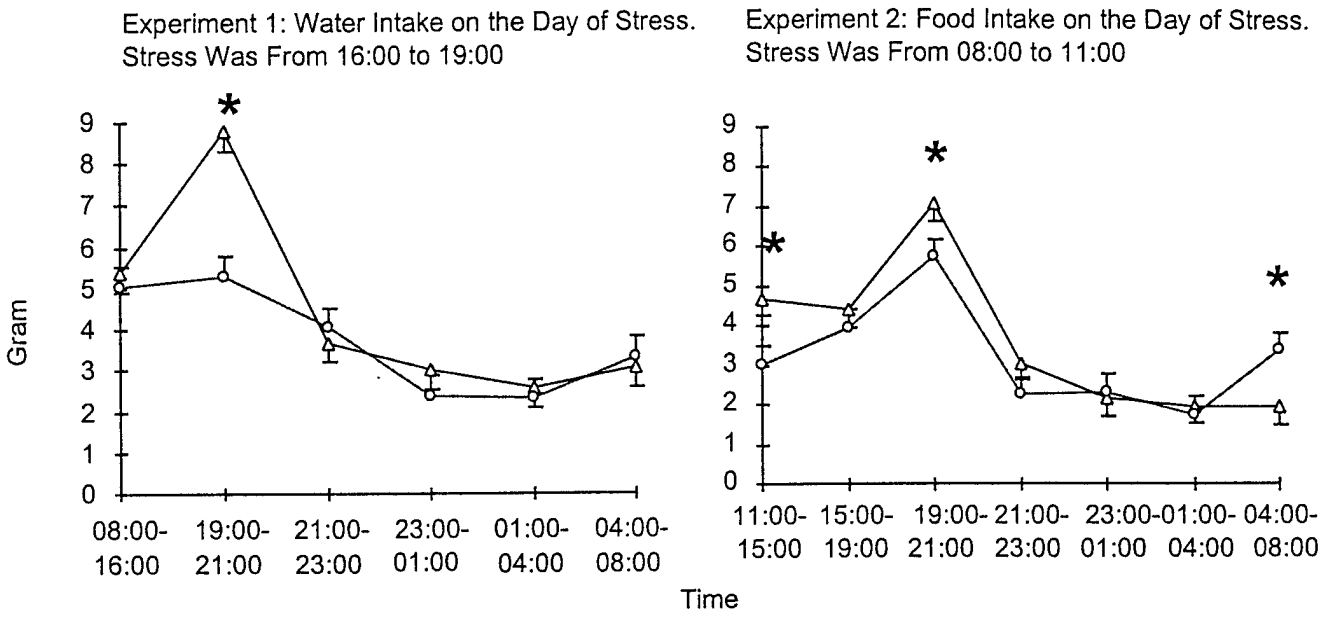
Data are means + sem for groups of 6 rats. Food intake and amino acid intake represent intake over 14 days. There was a significant effect of diet on both parameters, as indicated by superscripts.

Figure 8: Daily Food Intakes and Body Weights of Rats Restrained for 3 Hours at Different Stages of the Light Period

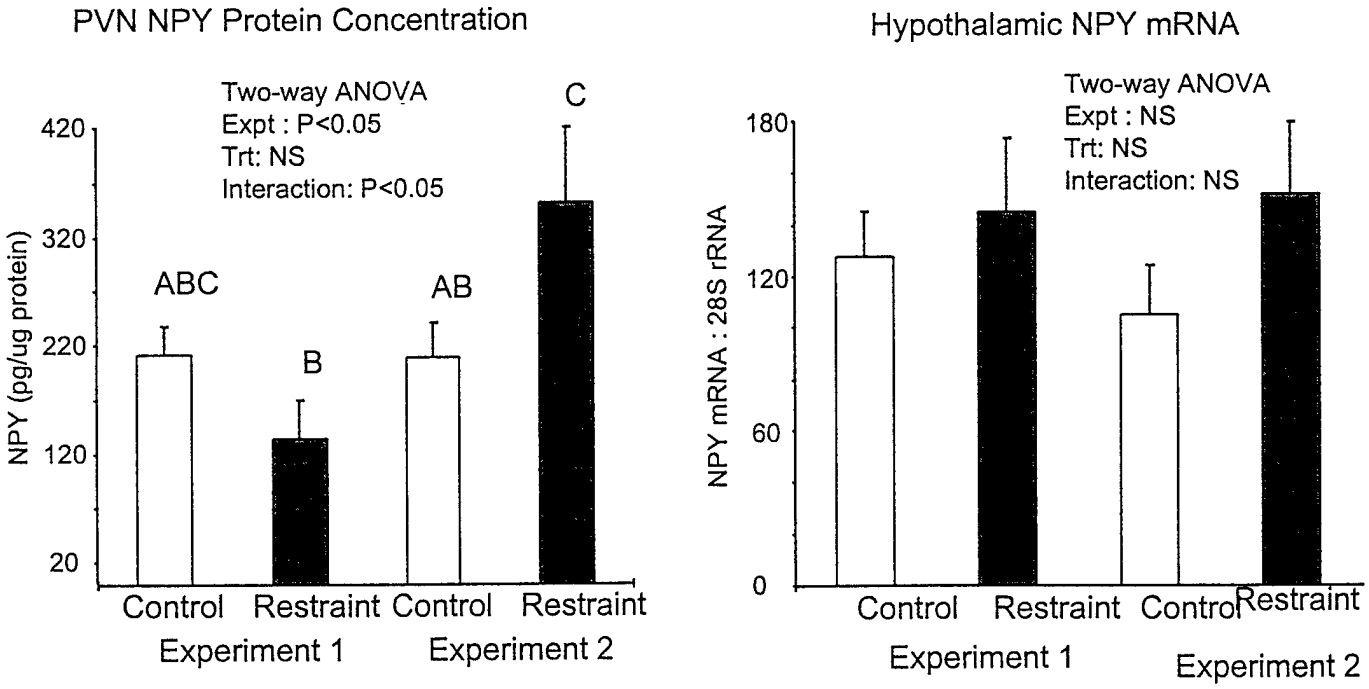


Data are means ± sem for groups of 16 rats. Significant differences, indicated by an asterisk were determined by repeated measures analysis. Rats were restrained for 3 hours on day 7. Stress significantly inhibited cumulative food intake during the recovery period when rats were stress at the end of the light period (expt 1) but not when rats were stressed at the start of the light period (experiment 2) ($P<0.07$). There was no significant effect of stress on cumulative water intake in either experiment.

Figure 9: Diurnal Food and Water Intake of Rats Restrained for 3 Hours at Different Stages of the Light Period

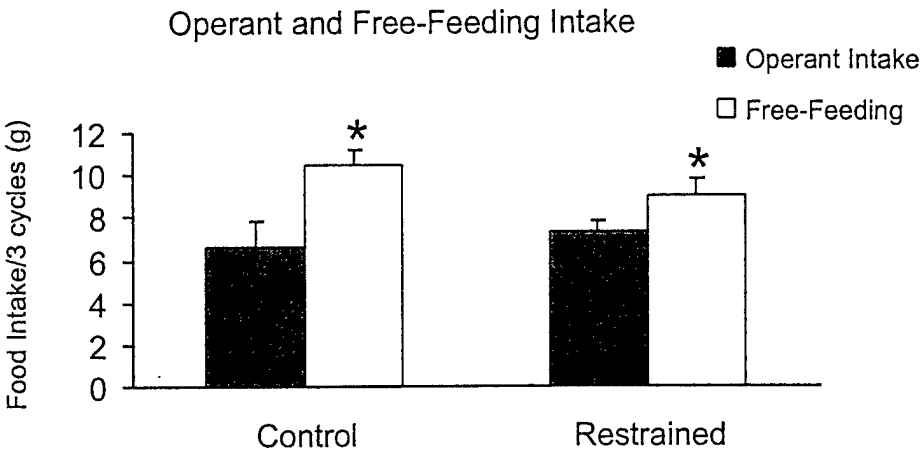


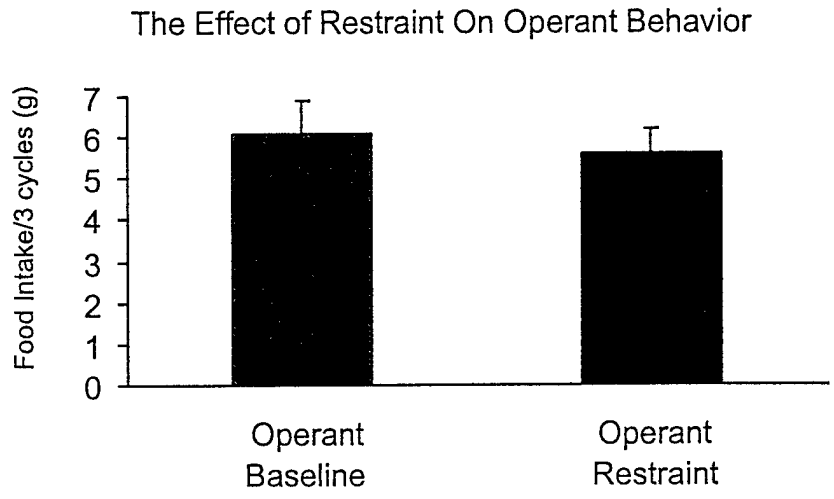
Data are means ± sem for groups of 16 rats. Significant differences, indicated by an asterisk were determined by repeated measures analysis. Rats were restrained for 3 hours prior to the first measures of intake. Stress caused significant reductions in food intake but not of water intake of the rats.

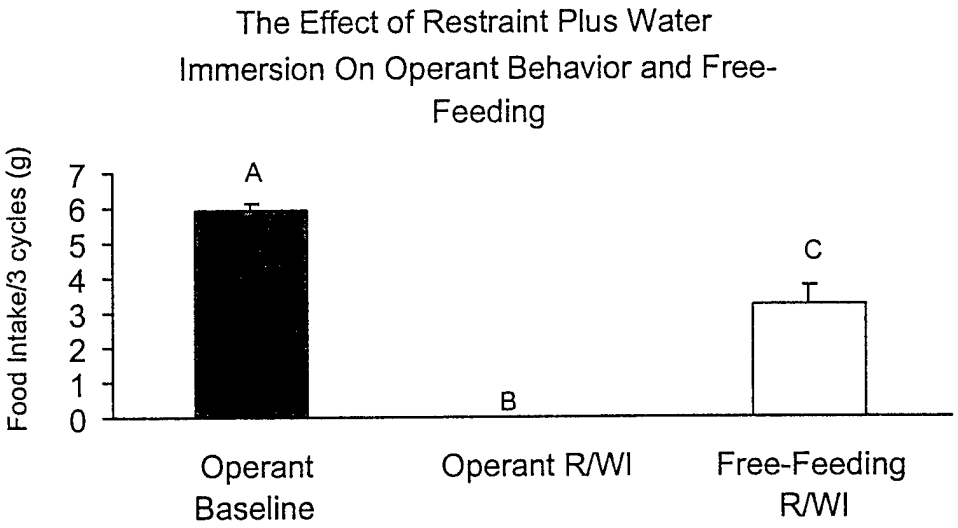


Hypothalamic NPY protein was measured by radioimmunoassay of protein extracted from PVN punches. There was a significant reduction in PVN NPY in rats restrained late in the day, compared with their controls, whereas there was a significant increase in rats restrained early in the day. There were no significant differences in NPY mRNA expression suggesting changes in protein release or turnover.

Figure 10: Operant and Free-Feeding Behavior of Food Restricted Rats





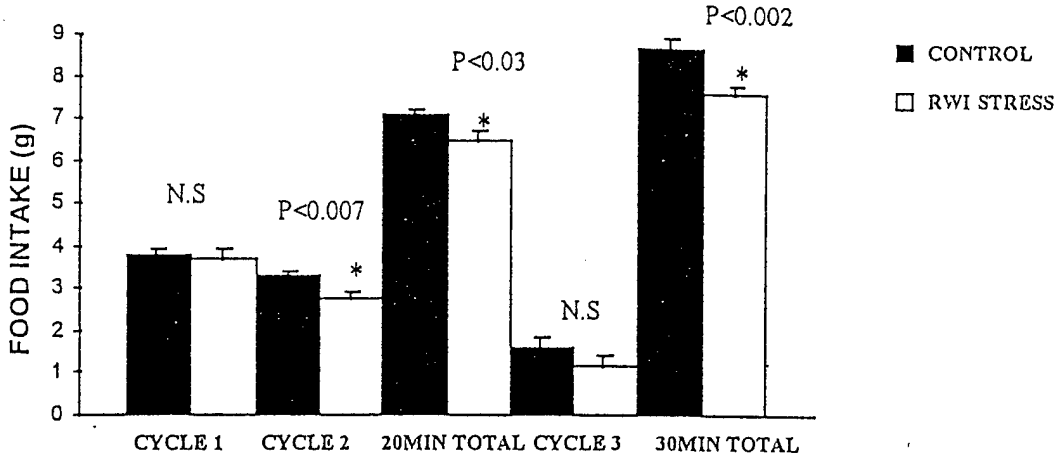


Data are means + sem for groups of 6 (expt 1) or 4 rats (expt 2 and 3). Significant differences in experiment 1 were determined by two-way ANOVA. Differences in Experiment 3 were determined by paired t-test.

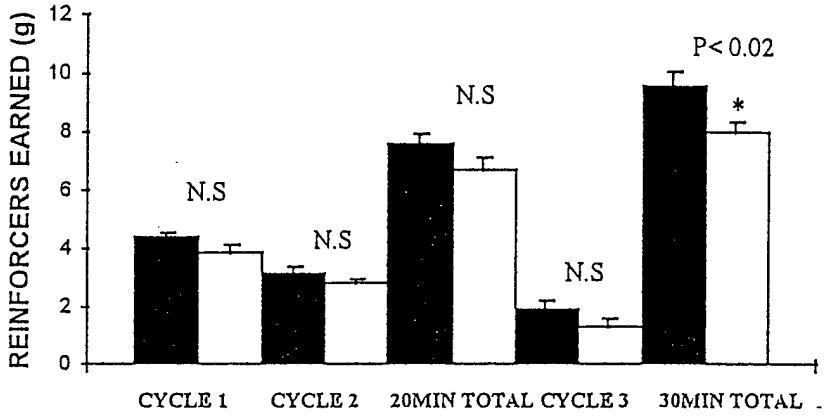
Figure 11:

30 MINUTES OF RWI STRESS

TEST 1: FREE-FEEDING FOOD INTAKE



TEST2 : OPERANT FR-5 BEHAVIOR



TEST2 : OPERANT FR-5 BEHAVIOR

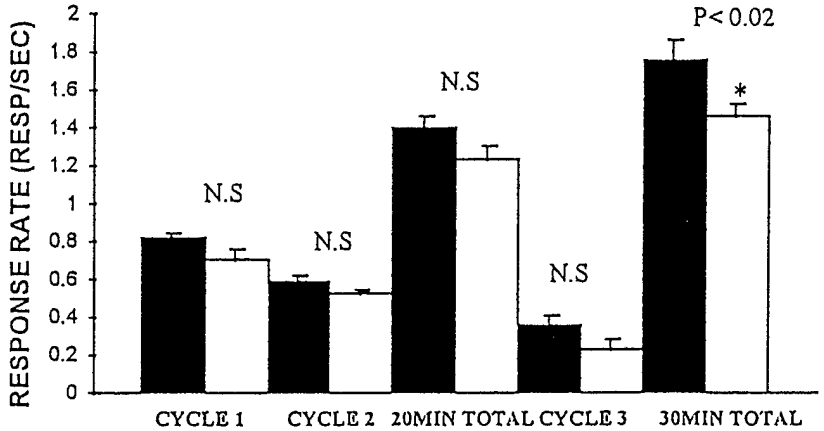


Figure 12:

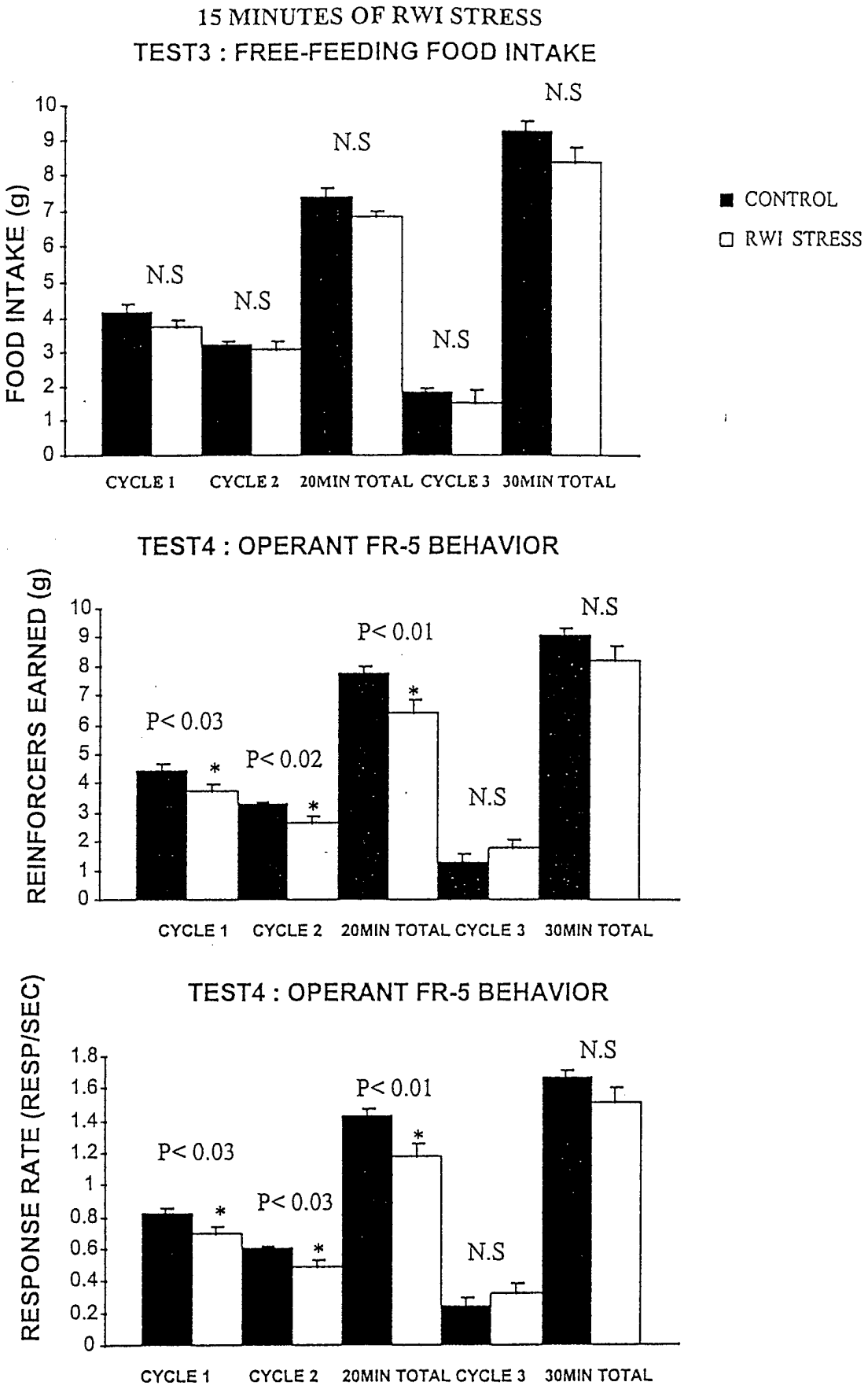
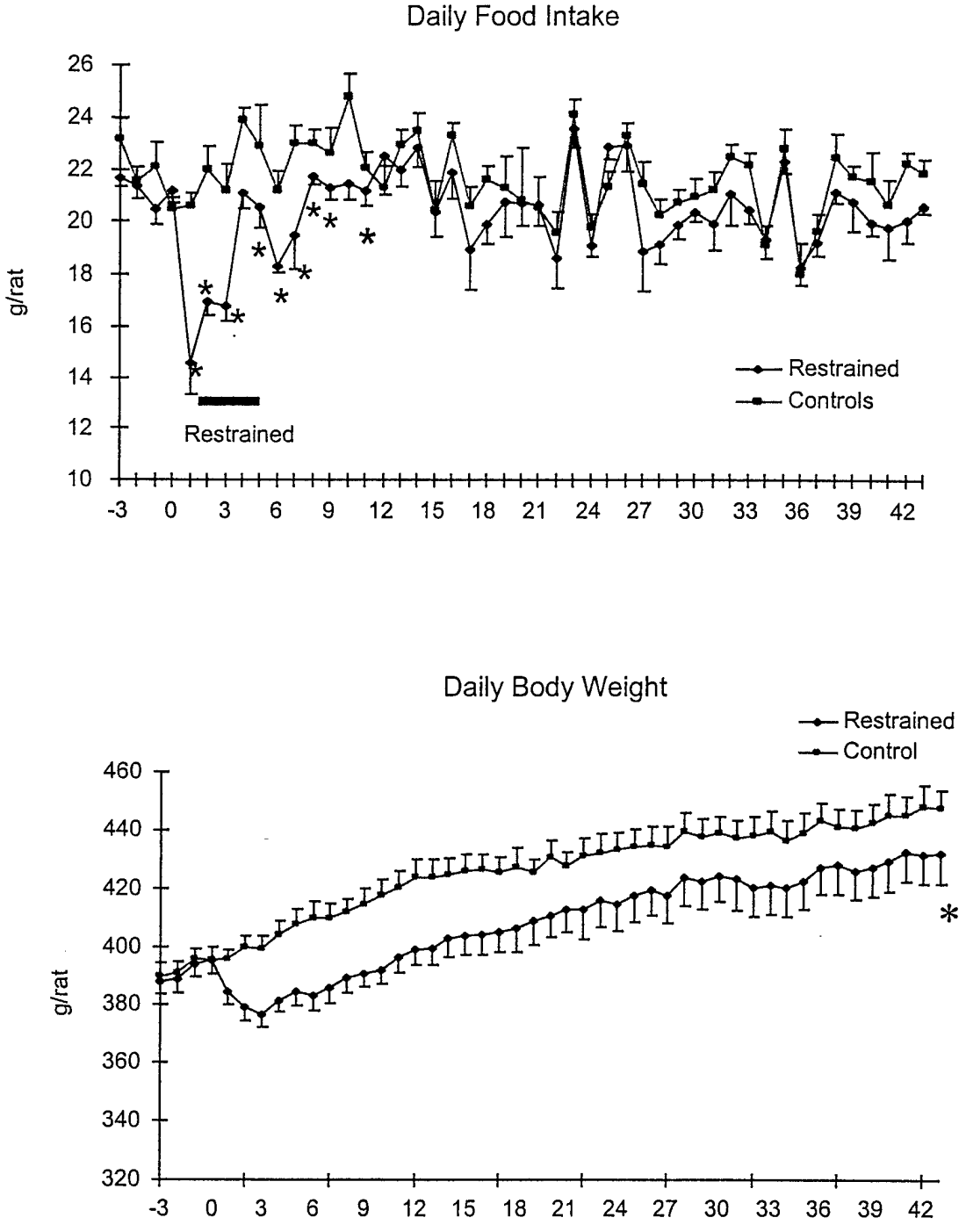
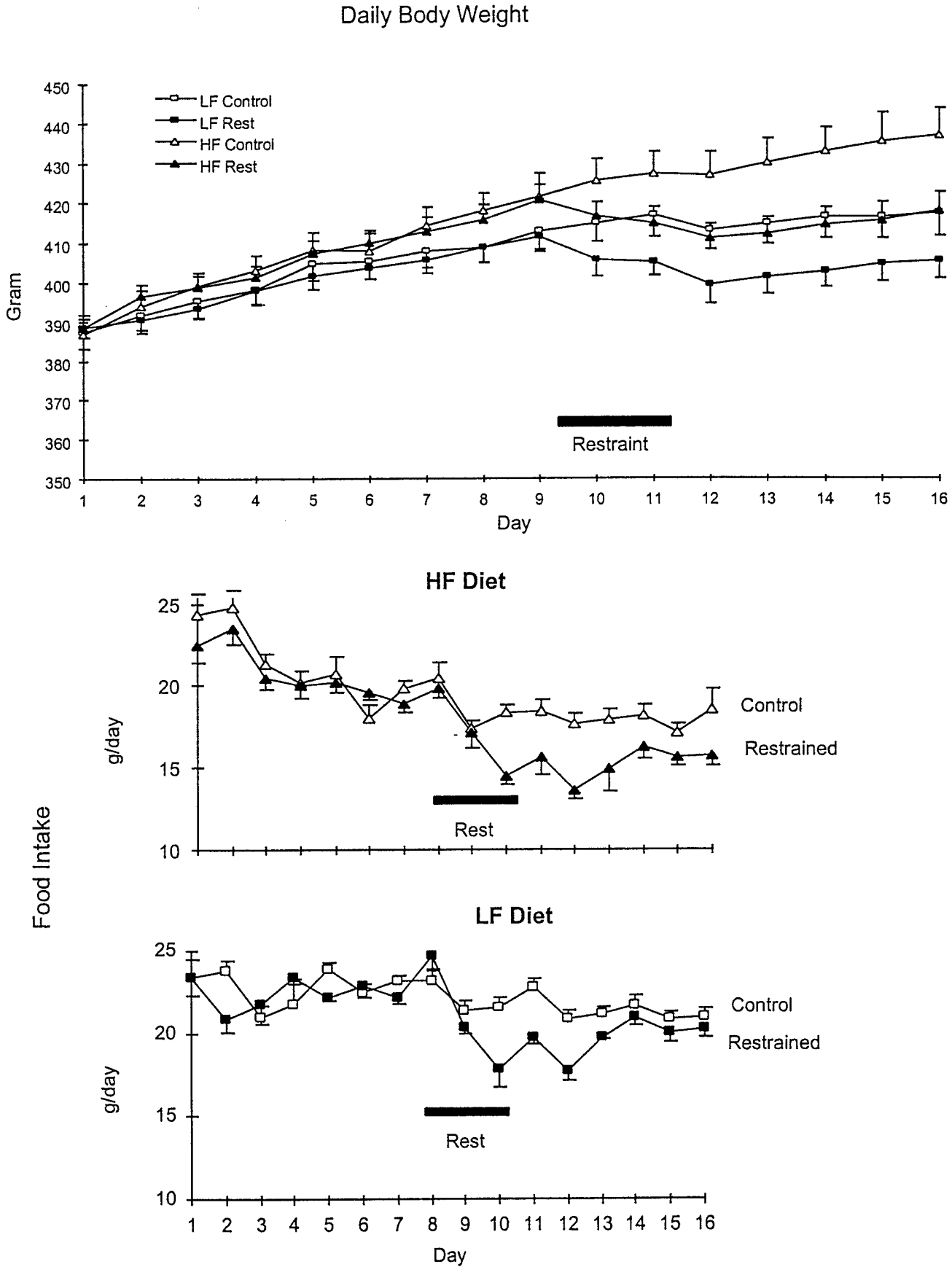

15 MINUTES OF RWI STRESS
TEST3 : FREE-FEEDING FOOD INTAKE
FOOD INTAKE (g)
CONTROL
RWI STRESS
N.S
N.S
N.S
N.S
N.S
CYCLE 1
CYCLE 2
20MIN TOTAL
CYCLE 3
30MIN TOTAL
TEST4 : OPERANT FR-5 BEHAVIOR
REINFORCERS EARNED (g)
P< 0.03
P< 0.02
P< 0.01
N.S
N.S
TEST4 : OPERANT FR-5 BEHAVIOR
RESPONSE RATE (RESP/SEC)
P< 0.03
P< 0.03
P< 0.01
N.S
N.S

Figure 13: Food Intakes abd Body Weights of Rats Restrained for 3 Hours on 3 Consecutive Days



Data are means ± sem for groups of 6 rats restrained for 3 hours on days 0, 1 and 2. Significant differences were determined by repeated measures analysis of variance An asterisk indicates a significant difference between control and restrained rats.

Figure 14: Food Intakes and Body weights of Rats Exposed to Repeated Restraint and Fed High or Low Fat Diets.



Data are means ± sem for groups of 8 rats killed 5 days after the end of repeated restraint.

Figure 15: Immune Measures In Rats Exposed to Repeated Restraint and Fed Low or High Fat Diet

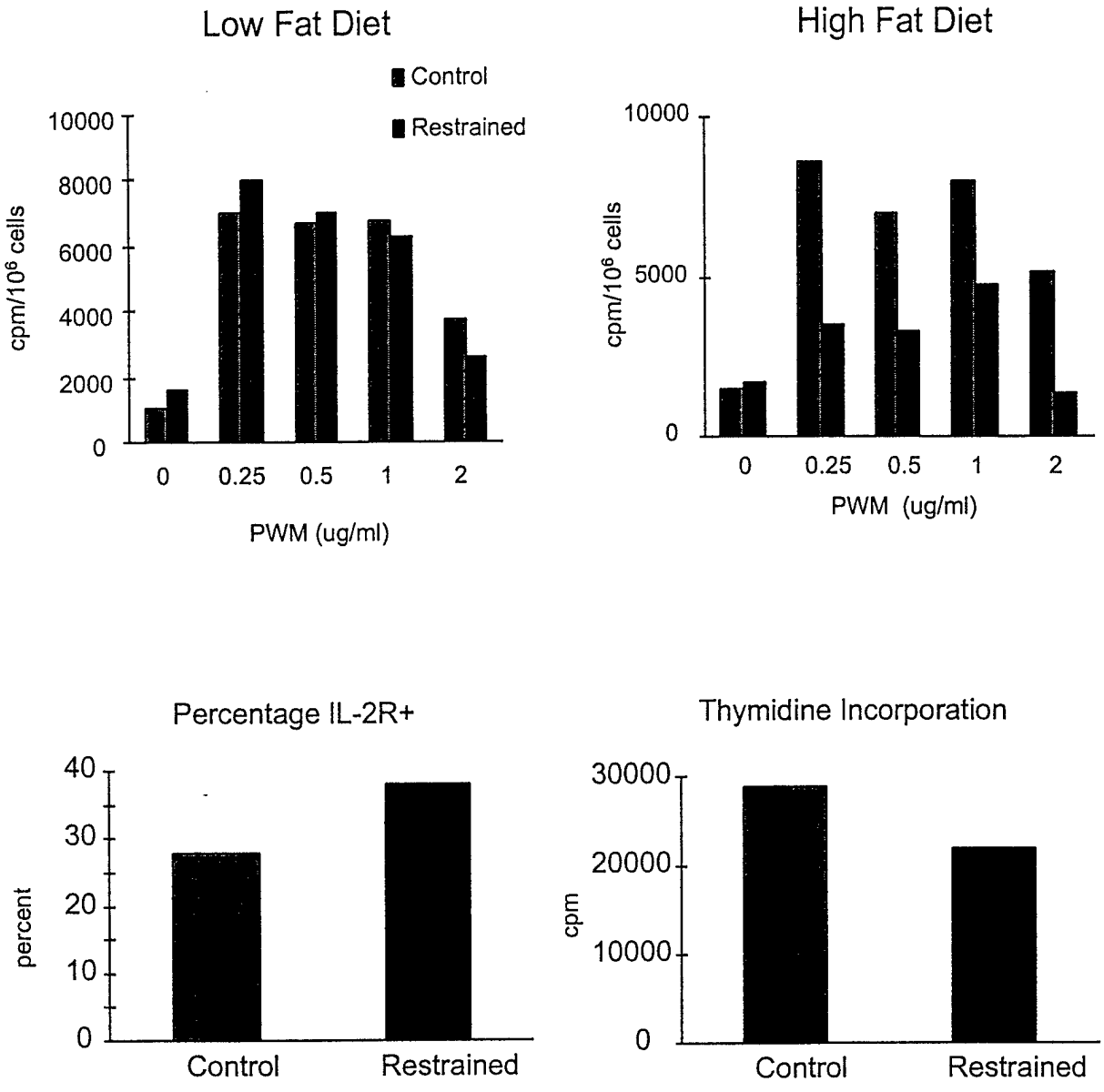
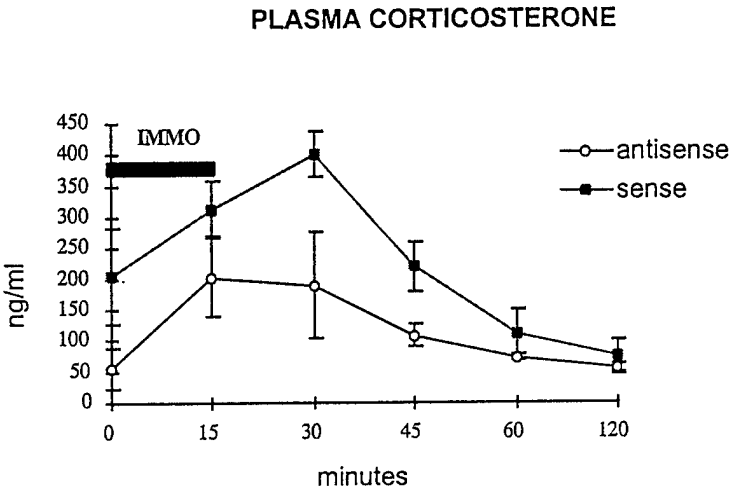
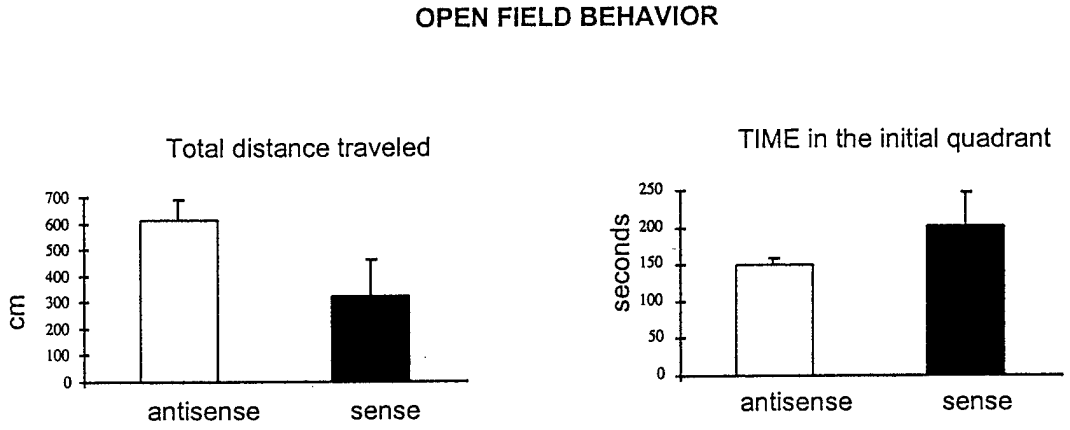


Figure 16:

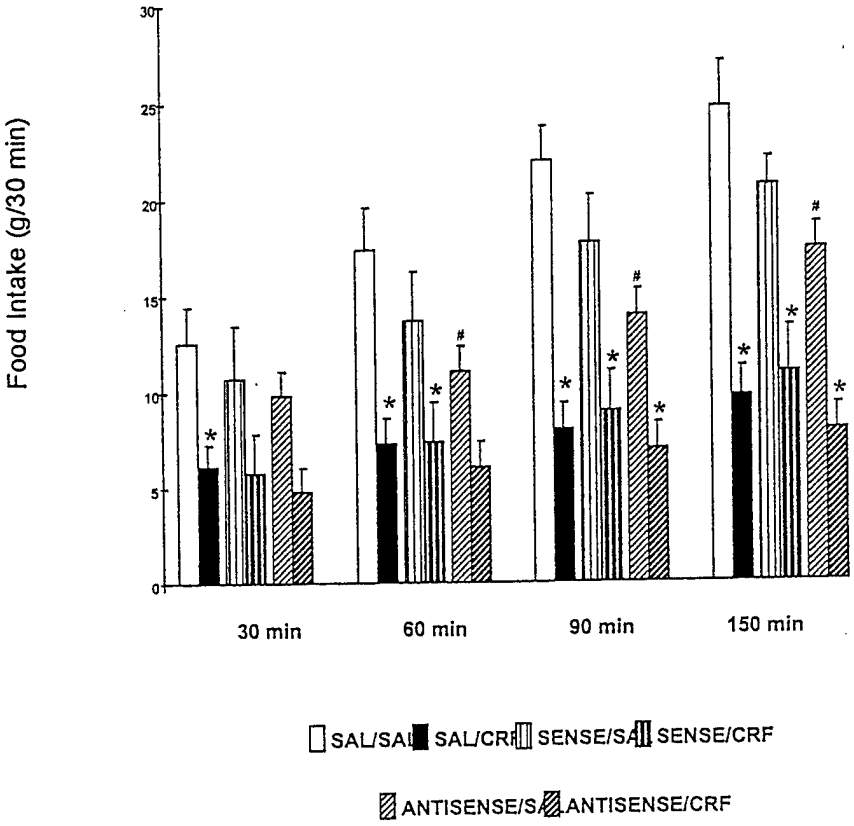
Antisense Oligonucleotide to CRF mRNA

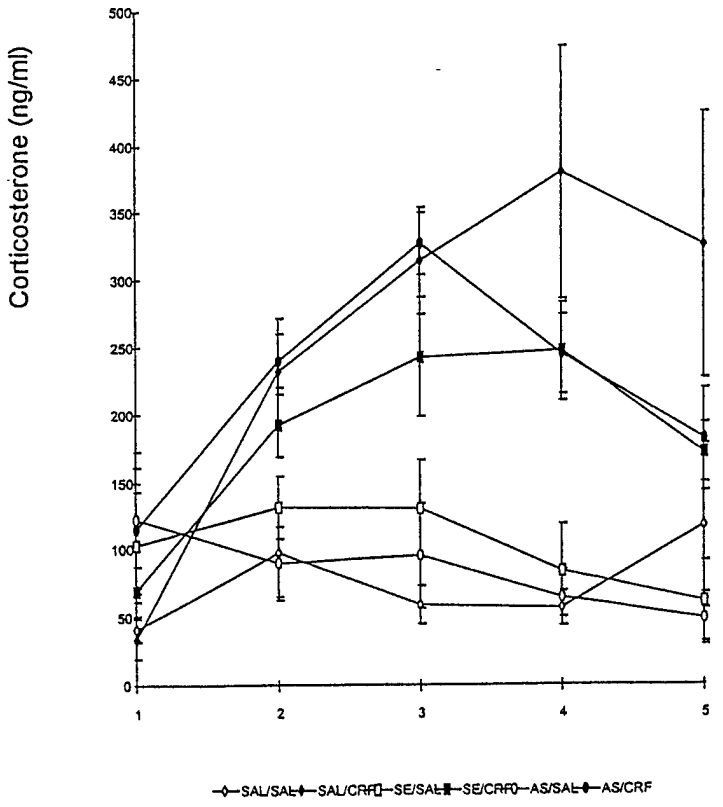




Data are means ± sem for groups of 3 rats

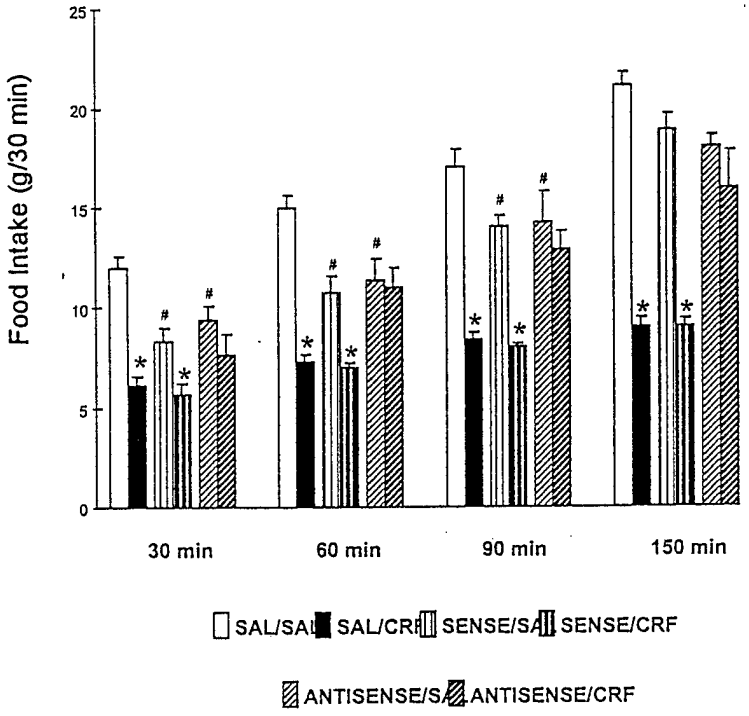
Figure 19: Rats treated With CRF1 Antisense

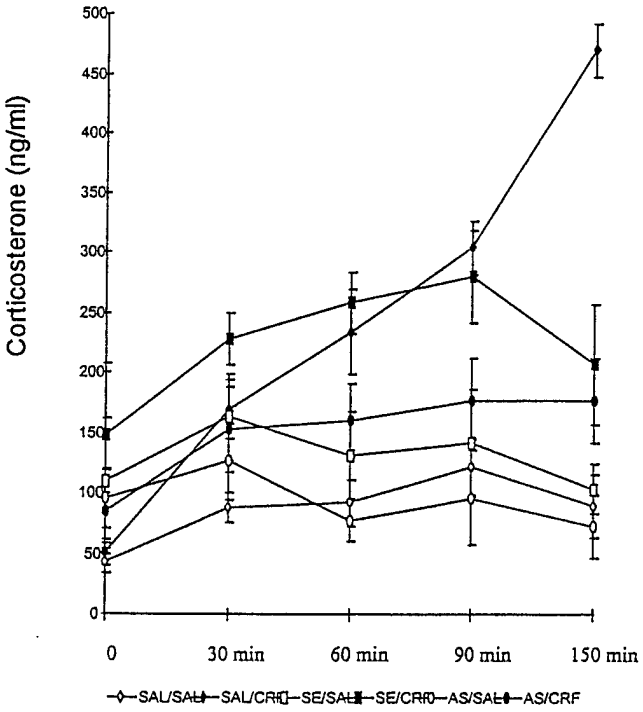




Data are means ± sem for groups of 6 rats.

Figure 20: Rats treated With CRF2 Antisense

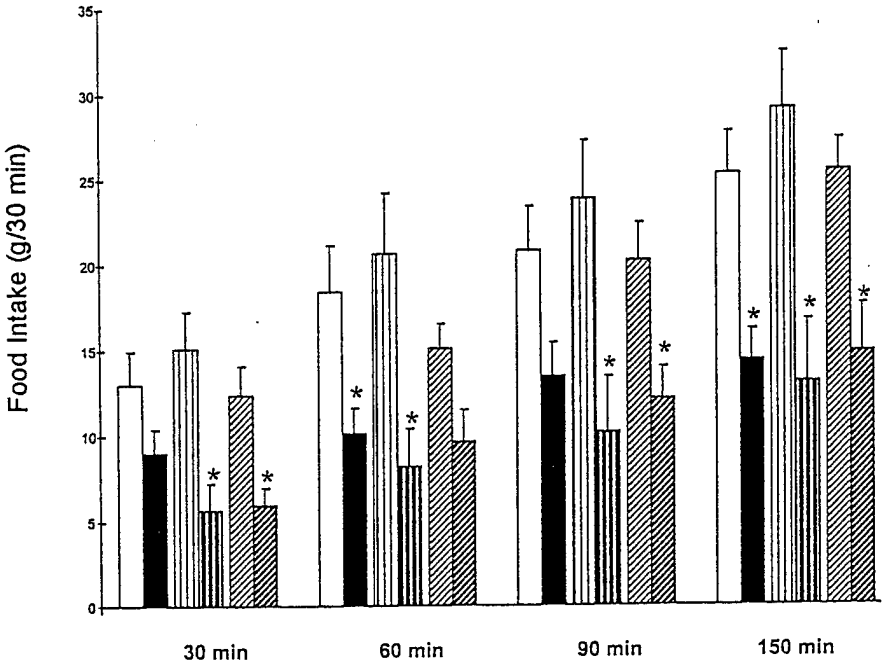




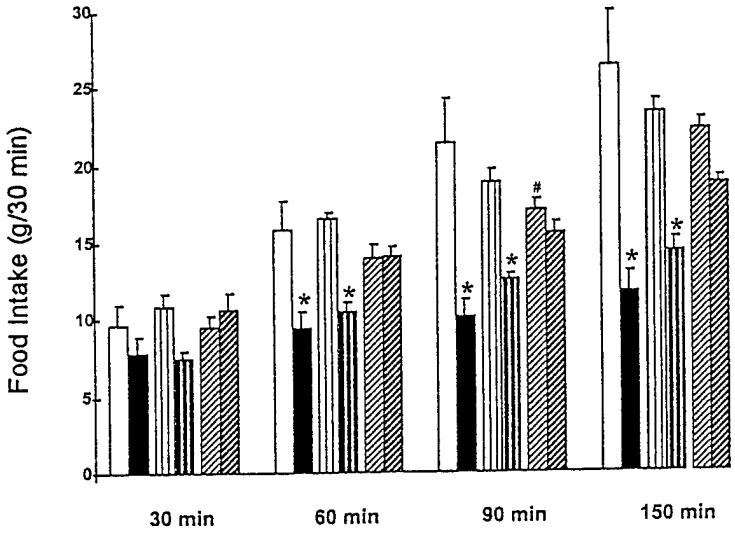
Data are means ± sem for groups of 6 rats.

Figure 21: Central Urocortin Infusion in Rats treated With CRF1 or CRF2 Antisense

CRF1 Antisense



CRF2 Antisense

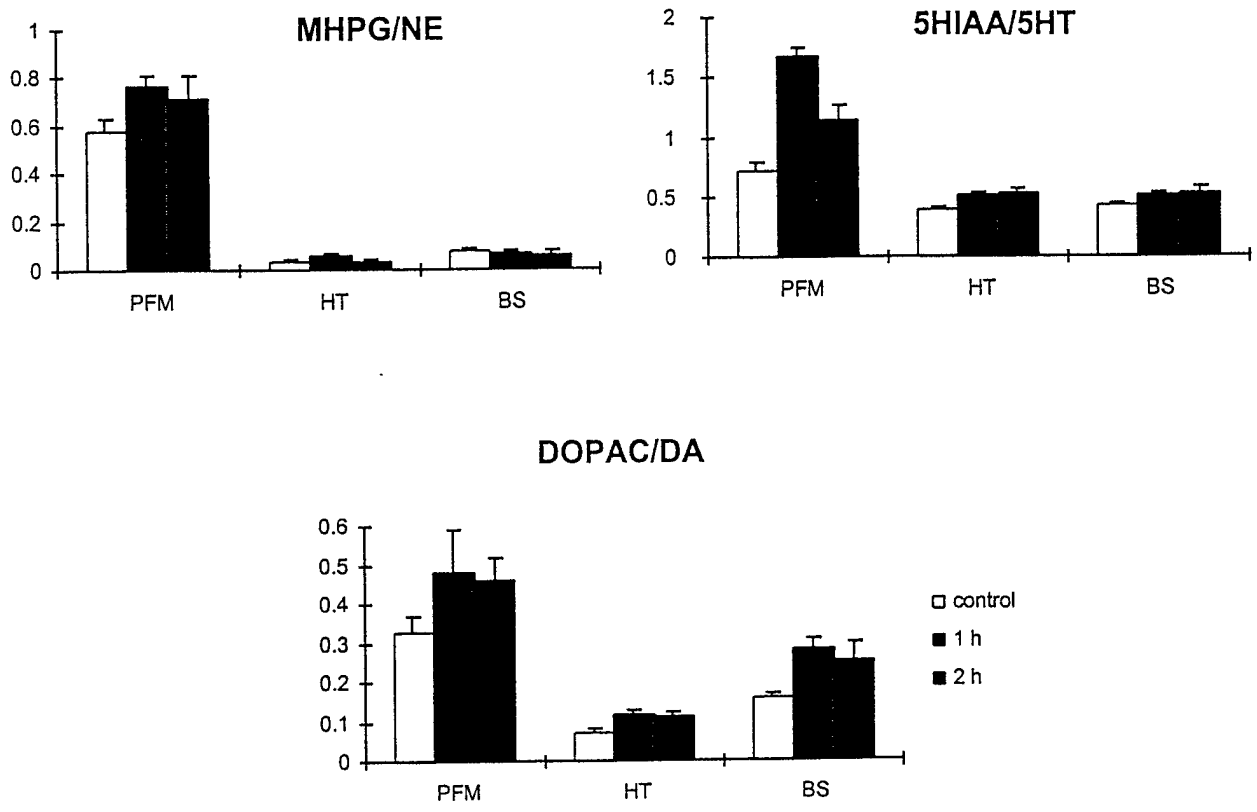


SAL/SAL SAL/CRF SENSE/SA SENSE/UROCORTIN

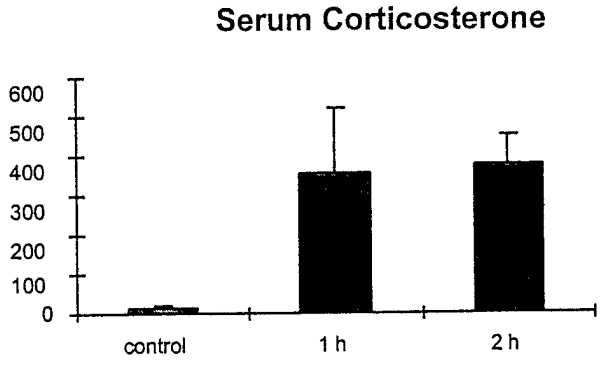
ANTISENSE/SA ANTISENSE/UROCORTIN

Data are means ± sem for groups of 6 rats.

Figure 22: The Effect of icv Urocortin on Serum Corticosterone and Hypothalamic Monoamines

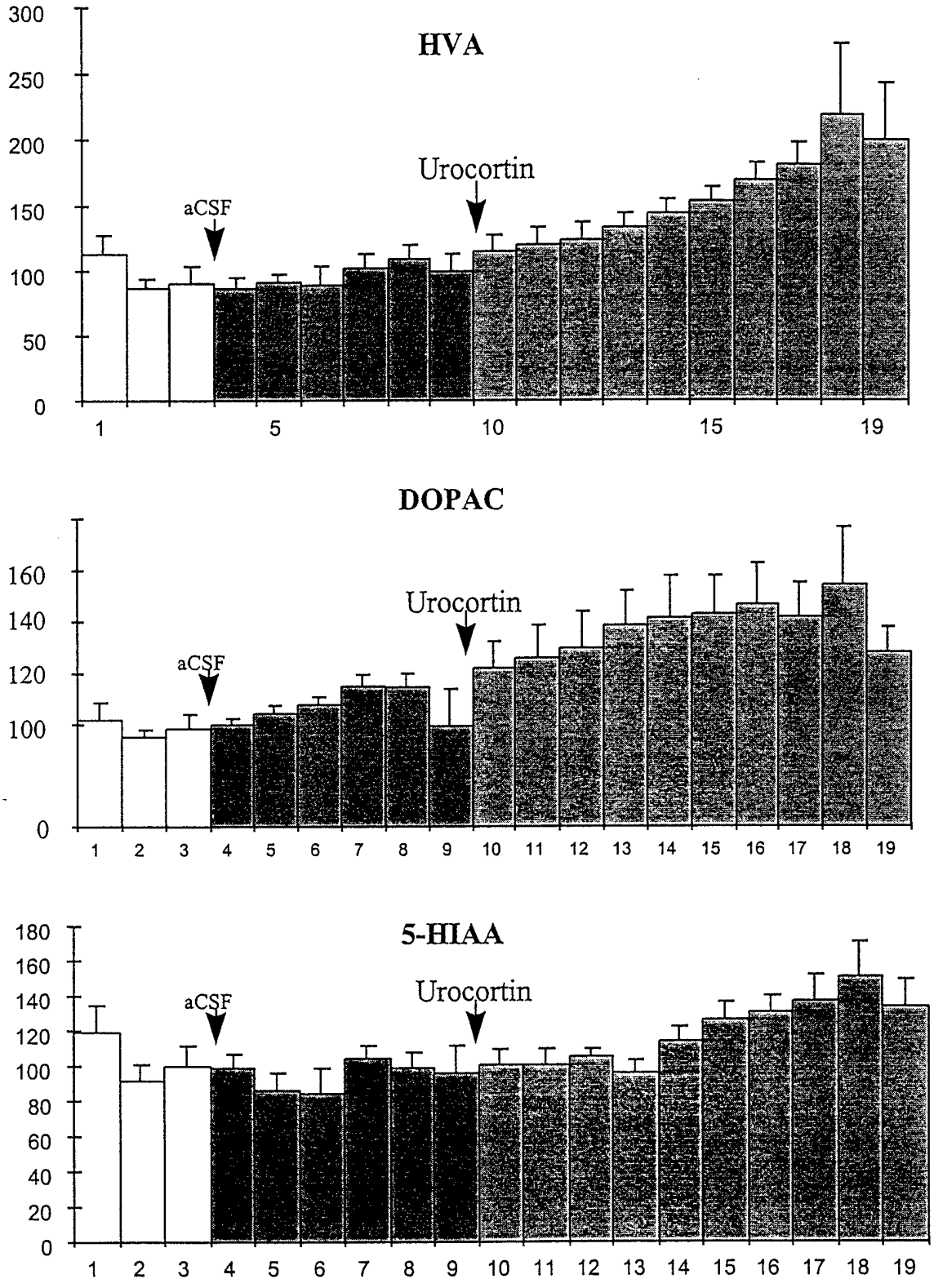


The effect of icv infusion of Urocortin (3 ug) on the norepinephrine, serotonin and dopamone metabolism in the prefrontal cortax (PFM), hypothalamus (HT) and brain stem (BS)



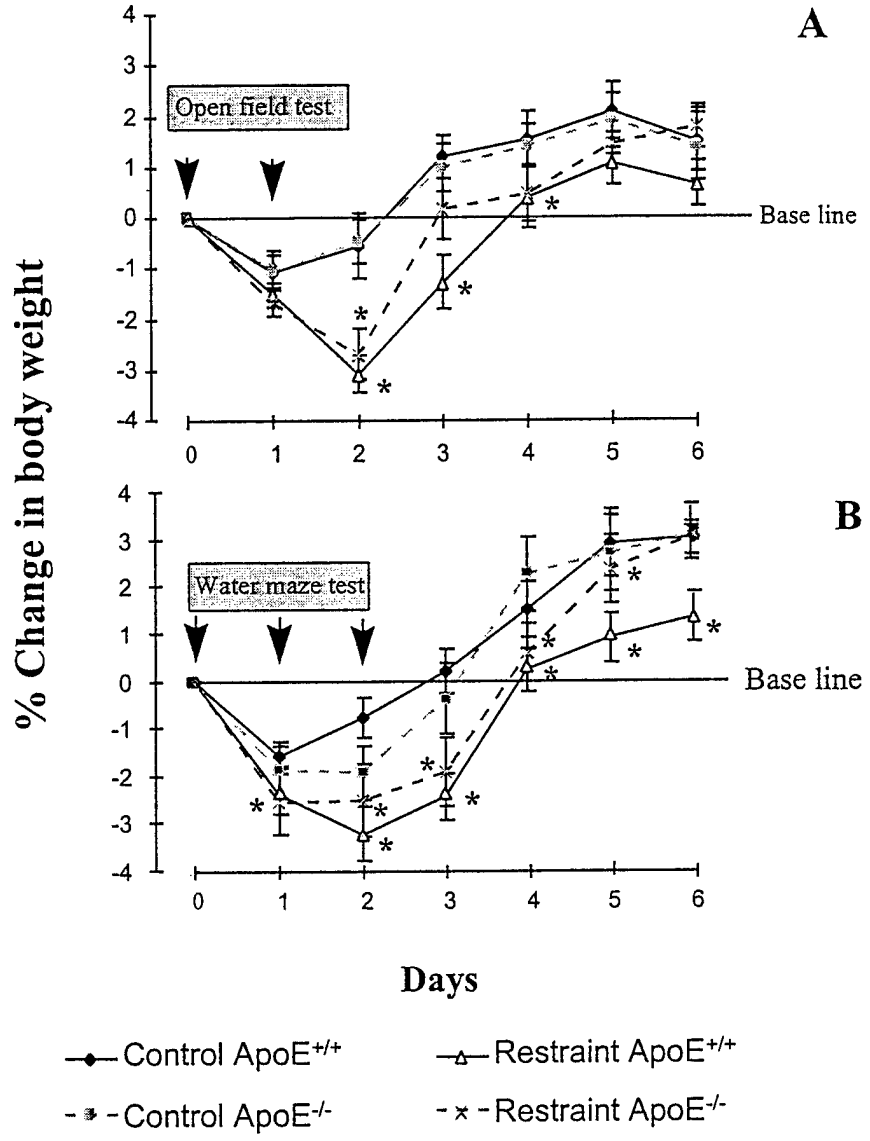
The effect of icv infusion of Urocortin (3 ug) on serum corticosterone

Figure 23: The Effect of icv Urocortin on Neurotransmitters in the Prefrontal Cortex



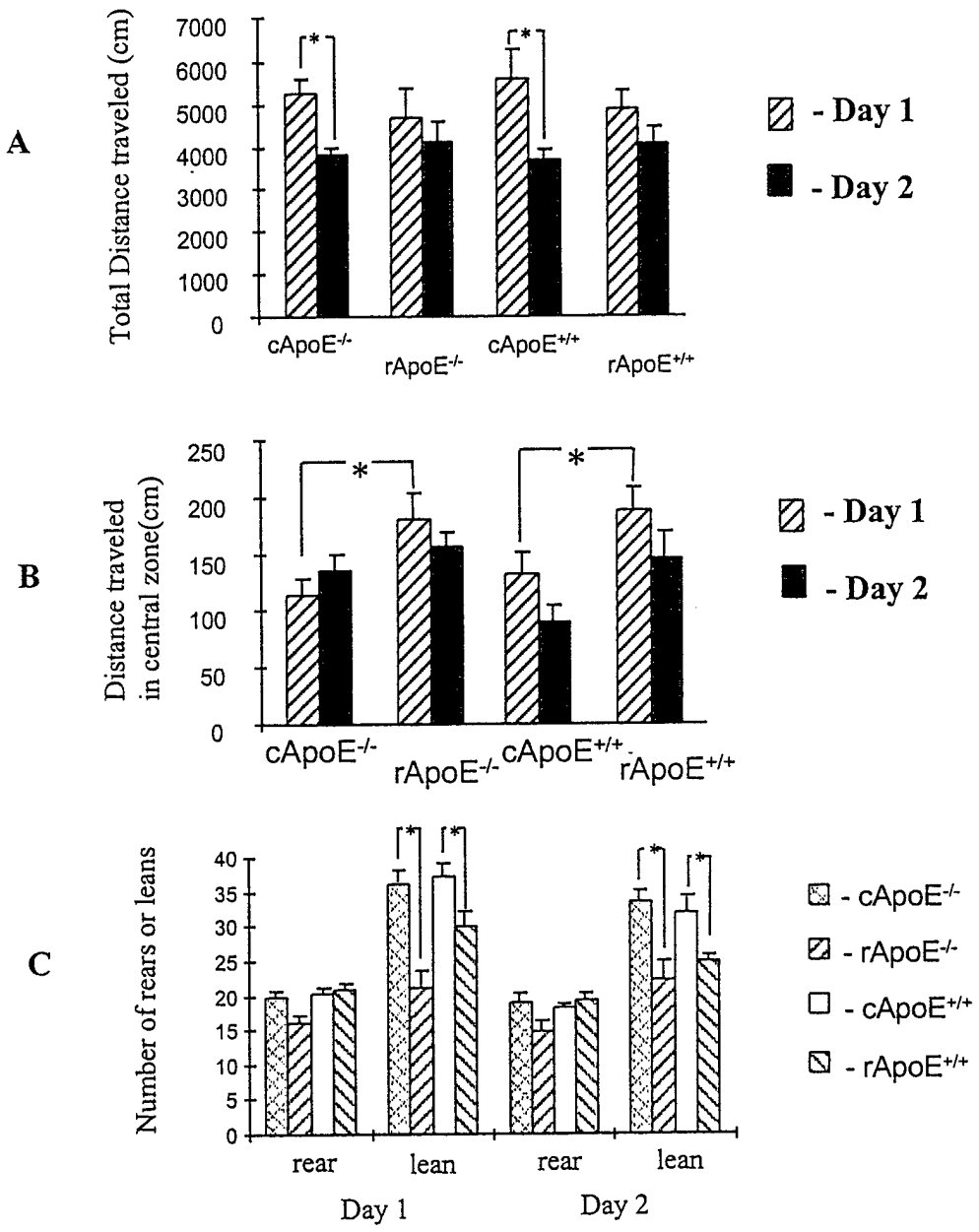
The effect of UCN (3 ug icv) on neurotransmitter concentrations in microdialysates collected from the medial prefrontal cortex. Data are presented as the mean of the percentage change from the baseline. Each bar represents a 20 min collection period.

Figure 24: Effects of stress on body weight of ApoE$^{+/+}$ and ApoE$^{-/-}$ mice



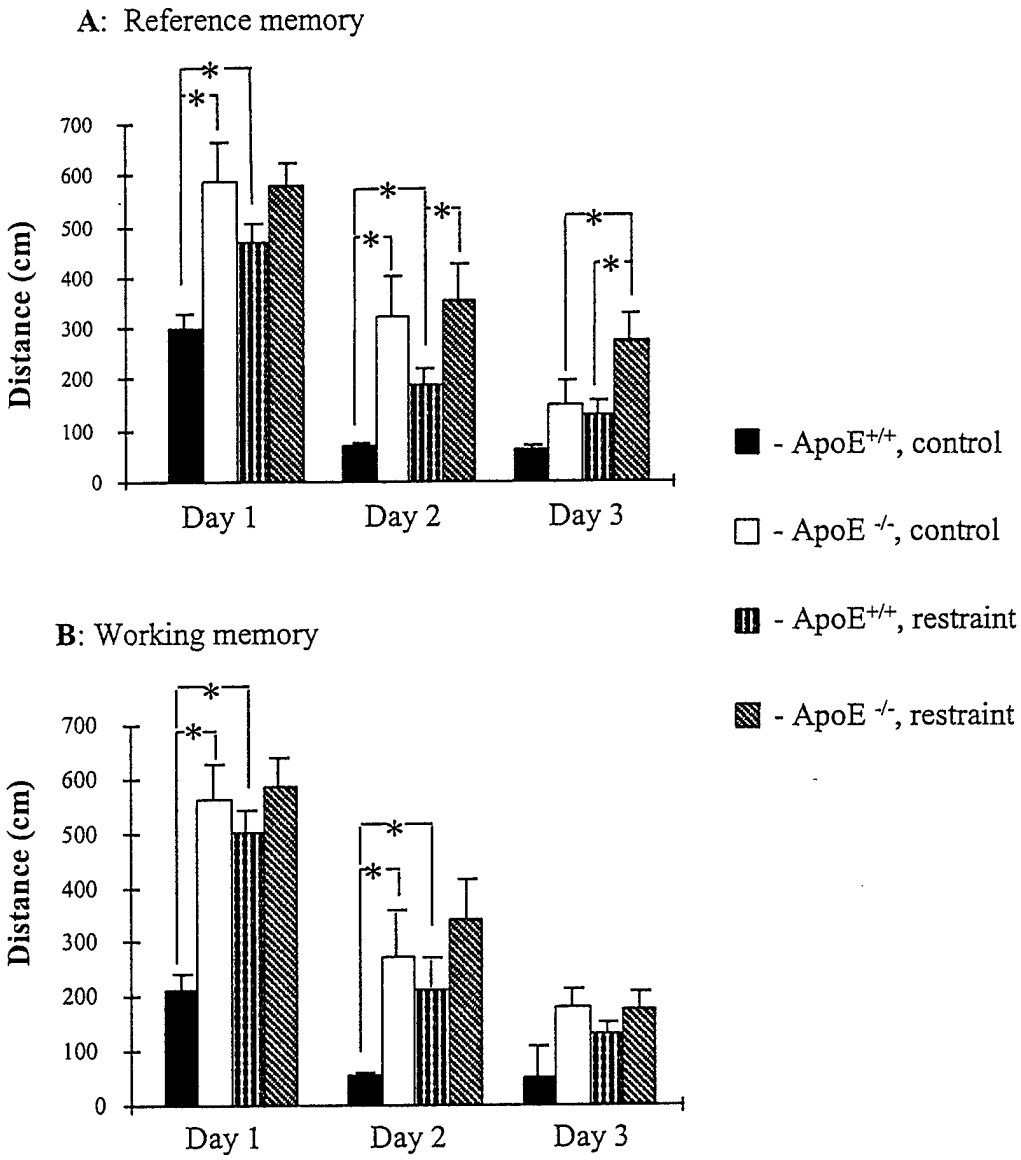
Daily body weight changes in response to restraint stress. The net changes were presented as a percentage of total body weight (as 100% base line) on the day before exposed to stress (Mean ± SEM, n=7). * indicates a significant difference ($p < 0.05$) between the restraint and control mice of the same genotype.

Figure 25: Effect of restraint on open-field activities of ApoE$^{+/+}$ and ApoE$^{-/-}$ mice



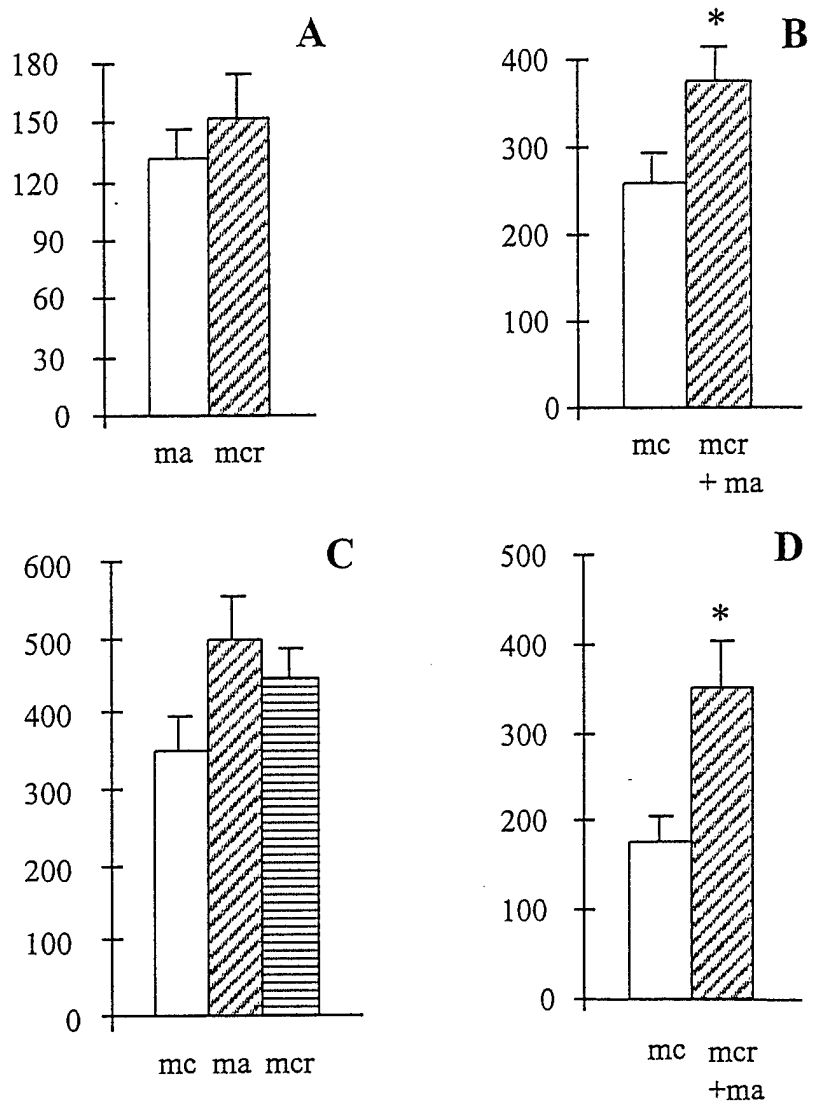
Distance traveled in the field (A) or central zone (B) and exploratory behaviors (C) by ApoE$^{+/+}$ or ApoE$^{-/-}$ mice were observed within the 5-min of open field test (Mean ± SEM, n=7). Both the control mice (cApoE) and those exposed a 20-min restraint (rApoE) were tested for 2 consecutive days. * indicates a significant difference ($p < 0.05$)

Figure 26: Memory impairments in ApoE-/- mice : Morris Water Maze task of mice with or without pre-exposure to restraint stress



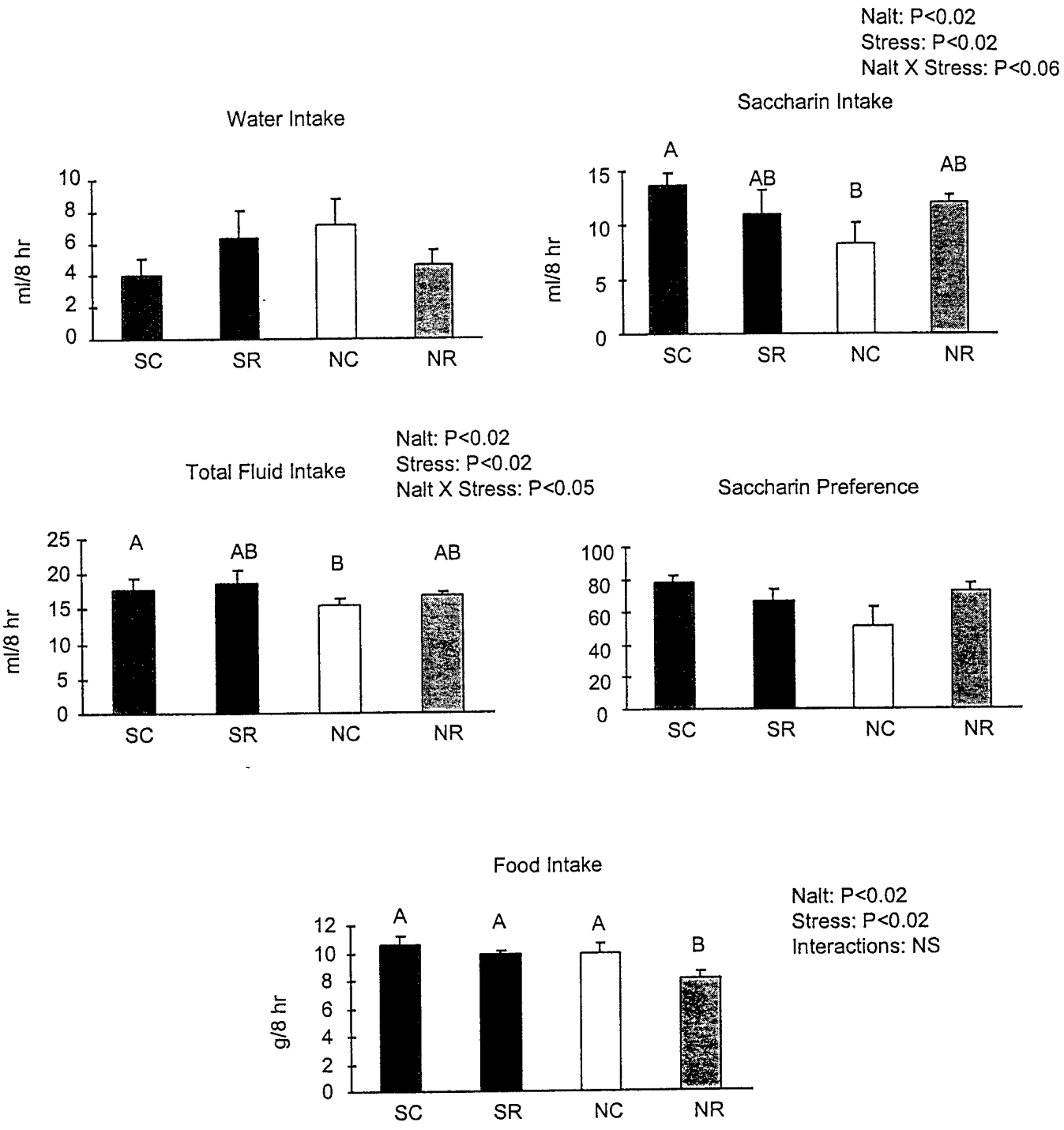
Data represent average distances traveled by mice to locate the platform (Mean ± SEM, n=7, pooled from 5 repeated measurements from different starting points). Mice were tested 2 trials (trial 1, reference memory; trial 2, working memory) per day for 3 consecutive days. Results were analyzed by MANOVA as described in the text.
* indicates a significant difference between the groups($p < 0.05$)

Figure 27: Effect of restraint stress on ApoE mRNA expression in mice



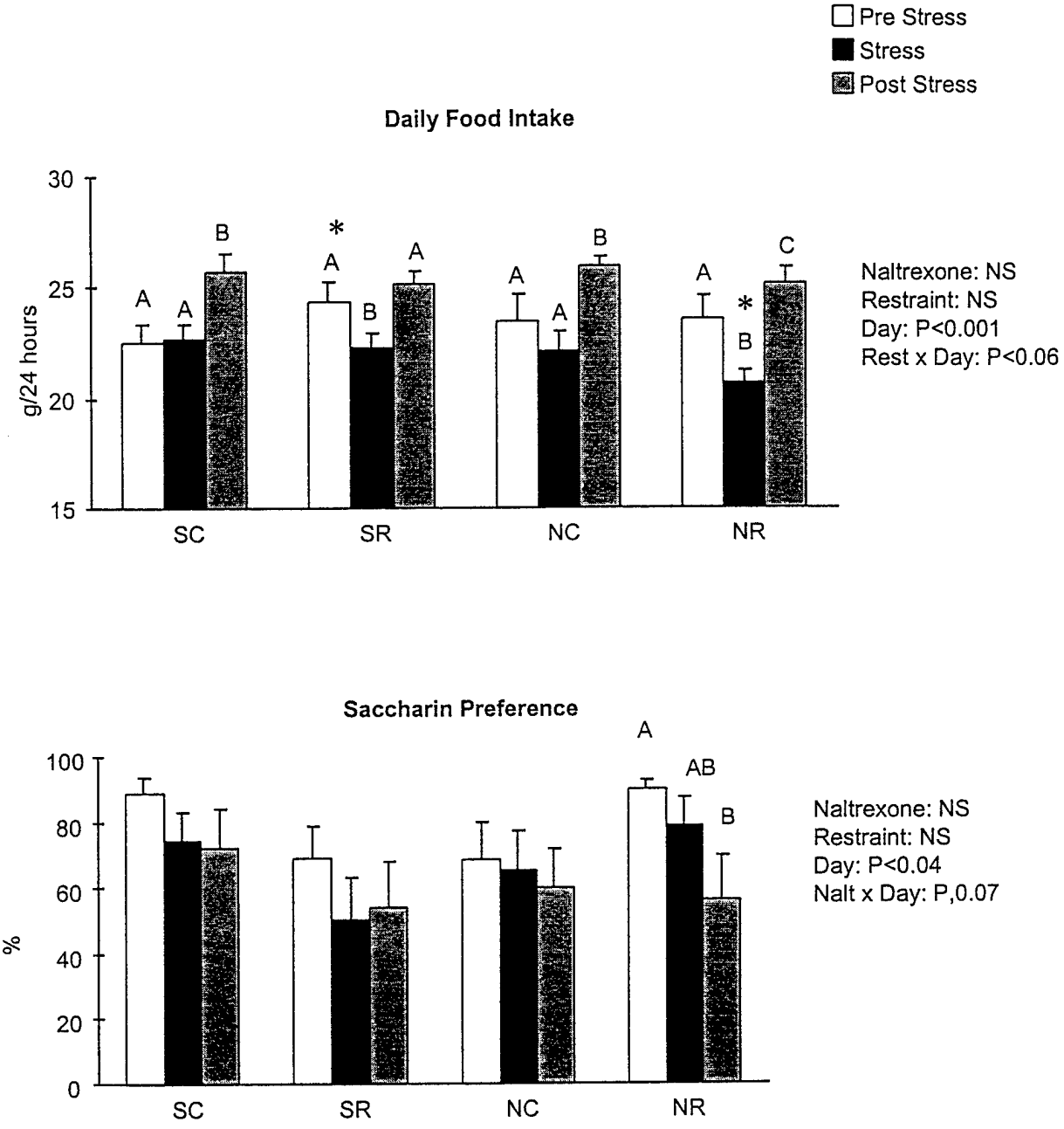
ApoE mRNA expression in hypothalamus (A-C) and liver (D), presented as the total points above background of ApoE/28s ratio (Mean ± SEM, n=6). Samples shown in Fig. A were obtained from Experiment #1, B and D from Experiment #2, and C from Experiment #3, as described in the text. (mc, non-restraint control; ma, restrained before killed; mcr, exposed to chronic restraint but not restrained before killed; mcr+ma, chronically stressed and restrained before killed). * indicates a significant difference between groups ($p < 0.05$)

Figure 28: Eight Hour Saccharin Preference and Food Intake in rats Restrained for One hour



Data are means ± sem for groups of 7 rats. SC = saline injected controls, NC = naltrexone injected controls, SR = saline injected restrained rats, NR = naltrexone treated restrained rats. Values for any given parameter that do not share a common superscript are significantly different at P<0.05.

Figure 29: Daily Food Intakes and Saccharin Preferences of Rats Exposed to One Hour of Restraint.



Data are means ± sem for groups of 7 rats. SC = saline injected controls, NC = naltrexone injected controls, SR = saline injected restrained rats, NR = naltrexone treated restrained rats. Values for any given parameter within a treatment group that do not share a common superscript are significantly different at P<0.05. An asterisk indicates a difference between a treatment group and saline injected controls on the same day.